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PROTEIN TYROSINE PHOSPHORYLATION IN CANCER DEVELOPMENT AND APPLICATIONS FOR RESEARCH

Sofie PIERAETS

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Cover illustration “Regulation of Protein Tyrosine Phosphorylation by Protein Tyrosine Kinases and Protein Tyrosine Phosphatases” designed by Somersault18:24

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LIST OF ABBREVIATIONS

ABL	v-abl Abelson murine leukemia viral oncogene homolog 1
ADAM	a disintegrin and metalloproteinase
ADP	adenosine diphosphate
AKAP8	a kinase (PRKA) anchor protein 8
AKT	v-akt murine thymoma viral oncogene
ALL	Acute lymphoblastic leukemia
AML	acute myeloid leukemia
ANKS1	ankyrin repeat and sterile alpha motif domain containing 1A
APC	antigen-presenting cell
ARHGAP27	Rho GTPase activating protein 27
ARHGAP30	Rho GTPase activating protein 30
ARP2/3	actin related protein 2/3
ATP	adenosine triphosphate
AURKA	aurora kinase A
B-ALL	B-cell acute lymphoblastic leukemia
BCR	breakpoint cluster region
BRAF	v-raf murine sarcoma viral oncogene homolog B
C	carboxy
Ca	Calcium
CBL	Cbl proto-oncogene, E3 ubiquitin protein ligase
CD	cluster of differentiation
CDC14	cell division cycle 14 phosphatase
CDKN2A/B	cyclin-dependent kinase inhibitor 2A/B
cDNA	complementary deoxyribonucleic acid
CGH	comparative genomic hybridization
CML	chronic myeloid leukemia
CNOT3	CCR4-NOT transcription complex, subunit 3
CNV	copy number variation
CPD	cyclobutane pyrimidine dimer
CRLF2	cytokine receptor-like factor 2
CSK	C-terminal SRC kinase
Ct	cycle threshold
db	database
DBNL	debrin-like
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DSP	dual specificity phosphatase
EGFR	epidermal growth factor receptor
EMT	epithelial-to-mesenchymal transition
ERK	extracellular signal-regulated kinase
EyA	eyes absent homologue
FAK	focal adhesion kinase
FAT	focal adhesion targeting
FBS	fetal bovine serum
FERM	four point one, Ezrin, Radixin and Moesin
FGF	fibroblast growth factor
FIP1L1	factor interacting with PAPOLA and CPSF1- like 1
FLT3	Fms-like tyrosine kinase 3
FNIII	fibronectin type III
FYN	FYN proto-oncogene
G1/2 phase	Gap 1/2 phase
GATA3	GATA binding protein 3
GBM	glioblastoma multiforme
gDNA	genomic DNA
GFP	green fluorescent protein
GRB2	growth factor receptor bound protein 2
GTP	guanosine triphosphate
H&E	hematoxylin and eosin
HCLS1	hematopoietic cell-specific Lyn substrate 1
HER2/neu	human epidermal growth factor receptor 2
HGS	hepatocyte growth factor-regulated tyrosine kinase substrate
HNRNPAB	heterogeneous nuclear ribonucleoprotein A/B
HRP	horseradish peroxidase
IFN	interferon
Ig	immunoglobulin
IKZF1	IKAROS family zinc finger 1
IL	interleukin
IL1RAPL1	interleukin 1 receptor accessory protein-like 1

IL2Rb	IL-2 receptor β
IL7-R	interleukin 7 receptor
ILK	integrin-linked kinase
INPP5D	inositol polyphoshate-5-phosphatase D
ITAM	immunoreceptor tyrosine-based activation motif
JAK	Janus kinase
kb	Kilobases
kDa	kiloDalton
KIT	v-kit hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
LAR	leukocyte common antigen-related
LAT	linker for activation of T-cells
LCK	lymphocyte-specific protein tyrosine kinase
LIMD1	LIM domains containing 1
LMW	low molecular weight
LOH	loss of heterozygosity
M phase	mitosis phase
MAM	mephrin/A5/RPTPμ
MAPK	mitogen-activated protein kinase
Mb	Megabases
MC1R	melanocortin 1 receptor
MDM2	mouse double minute 2 homolog
MEK	see "MAPK"
MET	MET proto-oncogene, receptor tyrosine kinase
MHC	major histocompatibility complex
MIM	missing-in-metastasis
miRNA	micro RNA
MOHITO	Mouse Hematopoietic Interleukin-dependent cell line of T-cell Origin
MPK	MAPK phosphatase
mRNA	messenger RNA
MSCV	murine stem cell virus
MYCN	v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog
N	amine
NCKIPSD	NCK interacting protein with SH3 domain
NCOA5	nuclear receptor coactivator 5
NEK1	NIMA-related kinase 1
NFAT	nuclear factor of activated T-cells
NGL-3	netrin-G ligand-3
NLS	nuclear localization signal
NOTCH1	NOTCH homolog 1
NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog
NRTK	non-receptor tyrosine kinase
NUP214	nucleoproin 214 kDa
PAG	phosphoprotein associated with glycosphingolipid microdomains
PAX5	paired box 5
PBX	pre-B-cell leukemia homeobox
PDGFRα/β	platelet-derived growth factor receptor alpha/beta
PEAK1	pseudopodiumm-enriched atypical kinase 1
PHF6	PHD finger protein 6
PI3K	phosphatidylinositol 3-kinase
PIK3AP1	phosphoinositide-3-kinase adaptor protein 1
PINCH1	particulary interesting new cysteine-histidine-rich 1
PKA	protein kinase A
PPIL1	peptidylprolyl isomerase (cyclophilin)-like 1
PPP1R12A	protein phosphatase 1, regulatory subunit 12A
PRL	phosphatases of regenerating liver
PSTPIP1	proline-serine-threonine phosphatase interacting protein 1
PTEN	phosphatase and tensin homolog
PTK	protein tyrosine kinase
PTK2B	protein tyrosine kinase 2 beta
PTP	protein tyrosine phosphatase
PTPN	protein tyrosine phosphatase, non-receptor type
PTPR	protein tyrosine phosphatase, receptor type
puro	puromycin
PVDF	polyvinylidene fluoride
qPCR	quantitative polymerase chain reaction
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RAS	Rat sarcoma viral oncogene homolog
RASA1	RAS p21 protein activator (GTPase activating protein) 1
RASGAP	RAS-GTPase
RAVER1	ribonucleoprotein, PTB-binding 1

RBM12B	RNA binding motif protein 12B
RGP	radial growth phase
RINL	Ras and Rab interactor-like
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
RPL5/10	ribosomal protein L5/10
RPMI	Rosswell Park Memorial Institute culture medium
RSU1	Ras suppressor protein 1
RTK	receptor tyrosine kinase
RUNX1	Runt-related transcription factor 1
S phase	synthesis phase
SD	standard deviation
SEM	standard error of the mean
SH2	SRC homology 2
SH3	SRC homology 3
shRNA	short hairpin RNA
SIFT	sorting intolerant from tolerant
siRNA	short interference RNA
SKAP1/2	src kinase associated phosphoprotein 1
Slitrk	SLIT and NTRK-like family
SLP-67	Src homology 2 domain-containing leukocyte protein of 76 kDa
SNAI1	Snail family zinc finger 1
SNP	single nucleotide polymorphism
SNV	single nucleotide variant
SNW1	SNW domain containing 1
SOCS	suppressor of cytokine signaling
SPF	specific pathogen free
SRC	v-src avian sarcoma viral oncogene homolog
STAT	signal transducer and activator of transcription
STRING	Search Tool for the Retrieval of Interacting Genes/proteins
SUZ12	SUZ12 polycomb repressive complex 2 subunit
t	translocation
T-ALL	T-cell acute lymphoblastic leukemia
TAL1	T-cell acute lymphocytic leukemia 1
TC-PTP	T-cell protein tyrosine phosphatase
TCGA	The Cancer Genome Atlas
TCR	T-cell receptor
TEC	tec protein tyrosine kinase
TLX1/3	T-cell leukemia homeobox 1/3
TNF	tumor necrosis factor
TP53	tumor protein p53
tPDGFRα	truncated PDGFRα
UTR	untranslated region
UV	ultraviolet
VEGFR	vascular endothelial growth factor receptor
VEP	Variant Effect Predictor
VGP	vertical growth phase
WASP	Wiskott-Aldrich syndrome protein
ZAP-70	zeta-chain associated protein kinase 70 kDa

LIST OF AMINO ACIDS

Full name	Three letter code	Single letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

SUMMARY

Protein tyrosine phosphorylation is an important signal transduction mechanism, which results in several cellular responses including proliferation, survival, differentiation, adhesion and migration. This process of protein tyrosine phosphorylation is a reversible posttranslational modification regulated by protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). Disruption of this tightly regulated process of protein tyrosine phosphorylation contributes to the uncontrolled growth of cells and the development of tumors.

In the first part of this manuscript, a screening system was developed to select functional shRNAs or siRNAs using the transforming capacity of a truncated form of the PDGFRα (tPDGFRα) receptor tyrosine kinase in the leukemic Ba/F3 cell line. In this screen effective shRNAs and siRNAs were identified targeting the *Pten* and *Cdkn2A* genes, which when cloned on the same transcript as the oncogenic *PDGFRα* resulted in a degradation of this transcript. This resultant loss of tPDGFRα blocked the proliferation of the Ba/F3 cells, which is a quantifiable and simple read-out for the effectiveness of each shRNA and siRNA. Additional advantages of this screening system are the independency of the obtained transduction efficiencies and the exclusion of toxic effects after the addition of the growth factor IL-3.

In the second part of the manuscript, we studied the role of PTPRD as a candidate tumor suppressor in acute lymphoblastic leukemia (ALL). We observed a high frequency of heterozygous deletions of *PTPRD* in the ALL samples, but found no correlation between the expression level and the copy number. This questions the role of PTPRD as a tumor suppressor in ALL. Indeed, often deletions across *PTPRD* locus also cover the region encoding the *CDKN2A* gene, a well-known tumor suppressor gene and may explain this current discrepancy. However, a role for PTPRD in hematopoiesis could not be completely discounted because of a high expression in undifferentiated hematopoietic cells and the spleen defects observed in the *Ptprd* knockout mice. Further we confirmed the STAT1 and STAT3 proteins as downstream signaling proteins of PTPRD in 293T cells. To further determine whether PTPRD is a tumor suppressor, its role in melanoma was further studied as previously had been shown that *PTPRD* is mutated in 12% of the melanoma cases. However, the expression levels of PTPRD were found to be low in melanoma samples and in melanocytes and did not correlate with the copy number. Moreover, the described mutations could more likely be passenger mutations as they are found across the entire gene in introns next to the exons further questioning its ascribed role as a tumor suppressor.

In the last part, we used phospho-proteomics to identify other signaling pathways controlled by PTPN2 next to the JAK/STAT pathway in T-ALL. Of the candidate proteins, the tyrosine kinase PTK2B was shown to be a bona fide downstream signaling protein of PTPN2 and was rapidly activated after IL-2 and IL-7 cytokine stimulation in leukemic cells. The PTK2B activation was found to depend on the SRC kinases and to be negatively regulated by PTPN2. Inactivation of PTK2B by siRNA-mediated knockdown or by SRC inhibitors had no effect on cell proliferation or migration.

SAMENVATTING

Proteïnen tyrosine fosforylatie is een belangrijk mechanisme van signaaltransductie, die verschillende cellulaire functies zoals proliferatie, overleving, differentiatie, adhesie en migratie controleert. Dit proces van proteïnen tyrosine fosforylatie is een omkeerbare posttranslationele modificatie die gereguleerd wordt door proteïnen tyrosine kinasen (PTKs) en proteïnen tyrosine fosfatasen (PTPs). Verstoring van dit nauw gereguleerd proces draagt bij tot de ongecontroleerde groei van cellen en de ontwikkeling van tumoren.

In het eerste deel van dit manuscript beschrijf ik de ontwikkeling van een screeningssysteem voor de selectie van functionele shRNA's en siRNA's door gebruik te maken van de transformerende capaciteit van een verkorte vorm van de PDGFRα receptor tyrosine kinase in de leukemie cellijn Ba/F3. Deze screen identificeerde werkzame shRNA's en siRNA's gericht tegen de *Pten* en *Cdkn2a* genen, welke wanneer gekloneerd op hetzelfde transcript als de oncogene *PDGFRα* zorgden voor de degradatie van dit transcript. Dit verlies van PDGFRα verhinderde de proliferatie van de Ba/F3 cellen, wat een kwantitatieve en gemakkelijke read-out is voor de selectie van werkzame shRNA's en siRNA's. Bijkomende voordelen van dit screeningssysteem zijn de onafhankelijkheid van de verkregen transductie efficiëntie en de mogelijkheid tot uitsluiten van toxische effecten na toevoeging van de IL-3 groeifactor.

In het tweede deel van dit manuscript bestuderen we de rol van PTPRD als een mogelijke tumorsuppressor in ALL. We observeerden een hoog aantal heterozygote deleties van *PTPRD* in ALL stalen, maar vonden geen correlatie tussen de gen expressie en het aantal kopijen. Dit stelde de rol van PTPRD als tumorsuppressor in vraag. Vaak omvatten de *PTPRD* deleties ook het *CDKN2A* locus, een welgekende tumorsuppressor, wat een verklaring kan zijn voor de wanverhouding tussen de expressie en het aantal kopijen. Nochtans kan de rol van PTPRD in de hematopoëse niet volledig worden uitgesloten, gezien de hoge expressie van PTPRD in ongedifferentieerde hematopoëtische cellen en gezien de vastgestelde defecten in de milt in de *Ptprd* knockout muizen. Verder bevestigden we STAT1 en STAT3 als neerwaarts gelegen signaalproteïnen van PTPRD in 293T cellen. Een mogelijke rol als tumorsuppressor van PTPRD werd verder bestudeerd in melanoma, aangezien eerder werd gerapporteerd dat *PTPRD* in 12% van de melanoma stalen gemuteerd is. De expressie niveau's van PTPRD waren echter zeer laag in melanoma stalen en in melanocyten en correleerden niet met het aantal kopijen. Daarenboven leken de beschreven mutaties eerder "passenger" mutaties te zijn door hun verspreide ligging over gans het gen in zowel intronen als exonen, wat de beschreven rol van PTPRD als tumorsuppressor verder in vraag stelt.

In het laatste deel gebruikten we phospho-proteomics om andere signaalwegen dan JAK/STAT te identificeren die door PTPN2 gereguleerd worden. Van de kandidaat proteïnen leek PTK2B een bonafide neerwaarts gelegen signaalproteïne te zijn van PTPN2, die snel geactiveerd werd na IL-2 en IL-7 cytokine stimulatie in leukemie cellen. Activatie van PTK2B bleek afhankelijk te zijn van de SRC kinasen en werd negatief gereguleerd door PTPN2. Inactivatie van PTK2B door siRNA-gemedieerde knockdown of door SRC inhibitors had echter geen effect of celgroei of -migratie.

CHAPTER I - INTRODUCTION

1 Cancer

The Belgian Cancer Register noted in 2011 a cancer incidence rate for females of 566,4/100000 persons/year and for males of 697,4/100000 persons/year. Furthermore, by aged 75, 1 in 3 men and 1 in 4 women will develop cancer. The current therapy for most cancer types is still chemotherapy, which causes severe side effects. In order to find new and more targeted therapies, it is essential to understand the multistep process of the development of cancer and its underlying molecular basis.

Cancer can arise in any tissue in the body and occurs when an otherwise normal cell acquires six core biological hallmarks to turn it into a tumor cell. These six core hallmarks of cancer were first described by Hanahan et al.[1] and include the ability of tumor cells to: 1) **Sustain continuous proliferation** through constitutive activation of proliferative signaling pathways or disruption of negative feedback mechanism of cell proliferation, 2) **evade growth suppression** through the inactivation of tumor suppressor genes, which normally turn-off proliferation signaling and control the cell cycle progression, 3) **resist cell death** or apoptosis by the loss of pro-apoptotic factors and the upregulation of anti-apoptotic factors, 4) **enable replicative immortality** through the extension of the telomeric DNA at the ends of the chromosome by the telomerase, 5) **stimulate angiogenesis** to provide access to nutrients and oxygen and removal of metabolic waste and carbon dioxide and 6) undergo **invasion and metastasis** whereby tumor cells alter their shape and lose their attachment to other cells and to the extracellular matrix (Fig. 1A)[2].

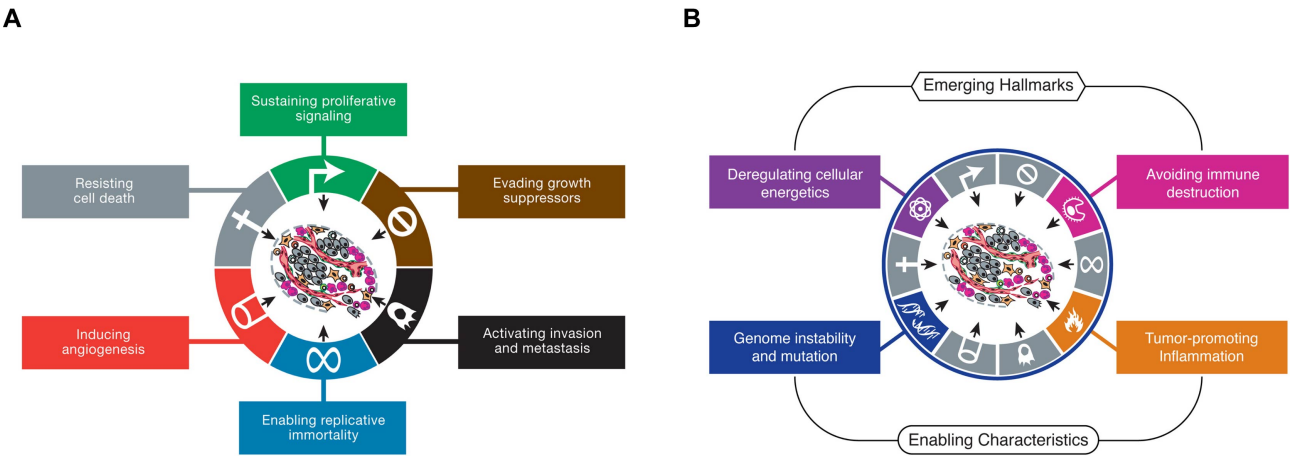


Figure 1. The hallmarks of cancer (A) Schematic representation of the six 'core' hallmarks acquired by a tumor cell during its multistep development. These six hallmarks are sustaining proliferation, evading growth suppression, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. (B) Furthermore tumor cells acquire two emerging hallmarks, reprogramming energy metabolism and avoiding immune system, and two enabling hallmarks, genome instability and tumor-promoting inflammation. (Adapted from reference[2])

In addition to these six 'core' hallmarks it is now becoming evident that the tumor cells can also acquire a number of newly described emerging and enabling hallmarks. The first one is the **reprogramming of their energy metabolism** where tumor cells use glycolysis to produce energy even under aerobic conditions. A second is the ability to **escape from the immune system**. A third is the accumulation of **mutations** and obtaining **genome instability**, which will result in the selection and expansion of subclones of the tumor cells. A forth is **tumor-promoting inflammation** where normal immune cells infiltrate and attack the tumor

cells, but is offset through the resulting inflammation promoting tumor growth by the delivery of growth, survival and proangiogenic factors (Fig. 1B)[2]. In addition a **block in differentiation** can be assigned as another hallmark of cancer, which is caused by the ectopic expression or loss of key transcription factors. This block in differentiation contributes to the development of hematologic malignancies by keeping the tumor cells in an immature state[3,4].

The underlying causes of these cancer hallmarks include both genetic and epigenetic alterations. Genetic alterations, such as deletion, amplification, insertion, translocation or point mutations, and epigenetic alterations, such as DNA methylation and histone modifications, change the expression levels of the affected genes or change their enzymatic activity with the above described consequences for the cell. The genetic alterations can be inherited or occur because of exposure to environmental factors, such as tobacco smoke, high-fat diet, toxic chemicals or viral infection. These genetic alterations will give the cells a selective advantage, which results in their outgrowth in the local tissue. Further accumulation of genetic alterations because of genome instability, can then promote tumor cells to acquire increased invasive and metastatic potential[2].

Of all these processes listed above, the ability of tumor cells to sustain continuous cellular proliferation can be achieved through increased protein tyrosine phosphorylation to activate downstream signal transduction pathways. This ability of tumor cells to increase protein tyrosine phosphorylation is further studied in this manuscript in the context of acute lymphoblastic leukemia and melanoma.

1.1 Acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is a malignant neoplasm arisen either from B-cell or T-cell lineage. B-cell acute lymphoblastic leukemia (B-ALL) is the most common subtype of ALL, while T-cell acute lymphoblastic leukemia (T-ALL) accounts for 15-25% of the ALL patients. With the current chemotherapy the cure rate is 70-80% in children but only 30-40% in adults. The prognosis of ALL patients with primary resistance and relapse is poor and it is necessary to identify new targets for therapy for these patients. In addition, despite the fact that most children with ALL survive due to effective chemotherapy regimens, the short term and long term side effects of chemotherapy are significant, and it remains of high interest to (partially) replace chemotherapy by more specific and less toxic targeted agents. A way to achieve this is to further understand the molecular basis of these leukemia – both at genetic and protein level. ALL is a complex disease, which is caused by a combination of genetic alterations including translocations, point mutations, deletions and epigenetic alterations. When some of these genetic alterations occur at the level of the hematopoietic precursor cells, it can cause cellular changes including increased proliferation and survival, cell cycle defects, impaired differentiation, unlimited self-renewal capacity and insensitivity to apoptotic signals. In regard to both B- and T-ALL, the genetic alterations, and clinical presentation of the disease can be markedly different and are further highlighted below[5,6].

1.1.1 B-cell acute lymphoblastic leukemia

B-ALL is a malignant neoplasm of the B-cell progenitors, which is characterized by hematologic abnormalities such as anemia, leukocytosis with neutropenia, thrombocytopenia and rarely eosinophilia. Other clinical presentations are lymphadenopathy, hepatosplenomegaly and infiltration in the central nervous system[5].

B-ALL cases possess a primary genetic alteration, often a chromosomal translocation, followed by the accumulation of secondary genetic alterations, affecting proteins involved in cell proliferation and differentiation. Chromosomal translocations found in B-ALL include t(1;19)(q23;p13.3) and t(9;22)(q34;q11). The t(1;19)(q23;p13.3) translocation results in the formation of a E2A-PBX fusion protein and occurs in 3-5% of the B-ALL cases. The E2A-PBX fusion protein functions as a transcriptional activator, which causes aberrant gene expression[5]. The t(9;22)(q34;q11) translocation results in the BCR-ABL1 fusion protein and occurs in 25% of the adult B-ALL patients. The BCR-ABL1 fusion protein acts as a constitutive active form of the ABL1 tyrosine kinase activity, which promotes cell proliferation[5,6]. The accumulation of secondary genetic alterations, stimulating cell proliferation and blocking B-cell differentiation, is necessary for driving the development of B-ALL. The activating mutations in the JAK proteins and the upregulation of the CRLF2 type I cytokine receptor through translocation are examples of proteins involved in cell proliferation pathways[5,6]. Deletion of the *CDKN2A/B* cell cycle regulators is present in 30-50% of the B-ALL patients. Deletions of the *PAX5* and *IKZF1* transcription factors, involved in B-cell development, are examples of genetic events affecting the B-cell differentiation[5-7].

1.1.2 T-cell acute lymphoblastic leukemia

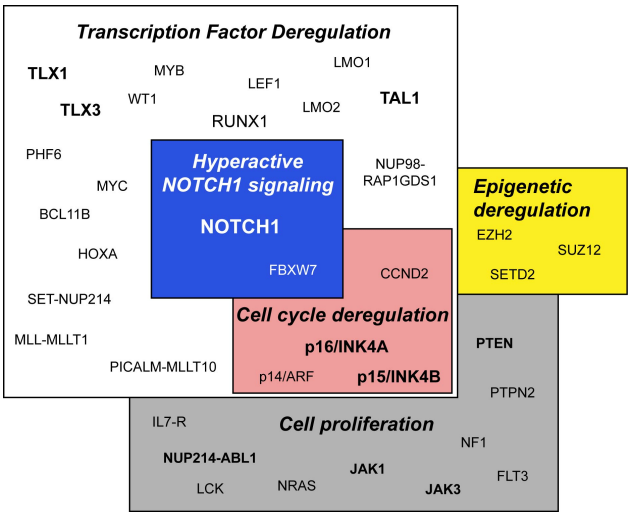


Figure 2. Schematic representation of the genetic defects in T-ALL Genetic lesions affecting the NOTCH signaling pathway and the CDKN2A/B cell cycle regulators are central in the pathogenesis of T-ALL. Furthermore, genetic alterations affecting cell differentiation, cell proliferation pathways and epigenetic regulators also contribute to the development of T-ALL. *(Adapted from De Keersmaecker, unpublished)*

T-ALL is an aggressive malignancy of thymocytes, which is characterized by high white blood cell counts, enlargement of mediastinal lymph nodes and frequent infiltration of the central nervous system. T-ALL accounts for 10-15% of the pediatric and 25% of the adult ALL cases and is more frequent in males than in females[3,4].

The genetic lesions found in T-ALL can be subdivided into five groups characterizing the disease (Fig. 2). The first and an important group in T-ALL genetic lesions is the one concerning the **NOTCH signaling pathway**. In 60% of the T-ALL samples NOTCH1 is activated through mutations and only in 1% of the cases by translocation. This uncontrolled activation of NOTCH1 contributes to the self-renewal capacity of the leukemic cells. The second group involves the

alterations in **cell cycle regulators**. Deletions of the *CDKN2A* locus are present in 70% of the T-ALL patients. *CDKN2A* contains the *p16INK4A* and *p14ARF* tumor suppressor genes, which are key regulators in the cell cycle. Alterations affecting **cell differentiation** are assembled into the third group and are often the result of ectopic expression of key transcription factors including TAL1, TLX1 and TLX3 and/or conversely the loss of RUNX1 and GATA3. The fourth group contains alterations in regulators of **chromatin structure** and includes mutations or deletions of the epigenetic regulators *SUZ12* and *PHF6*. Genetic lesions in components of **signaling pathways** are ranked together into the fifth group. Alterations in this group mainly affect cell growth and lineage commitment of the T-cell progenitors[3,4]. These can include activating mutations in the IL7R-JAK/STAT pathway (present in 10% of T-ALL cases) or the loss of the tumor suppressive protein tyrosine phosphatases such as PTPN2 and PTPRD, which are further described in this doctoral thesis.

1.2 Melanoma

Melanoma is an aggressive malignancy of the melanocytes. During development melanocytes migrate from the neural crest to the basal layer of the skin. With their long dendrites melanocytes are connected with the keratinocytes located in the epidermis. Melanocytes produce the melanin pigment, which is transported in melanosomes to the keratinocytes. Melanin protects the keratinocytes by absorbing and scattering UV radiation. A low pigmentation and high sun exposure increase the incidence of melanoma[8].

The development of metastatic melanoma is a multistep process starting in the melanocytes. Increased proliferation of the melanocytes results in the development of benign nevi or dysplastic nevi[8]. Metastatic melanoma originates in 75% of the cases from transforming melanocytes and only in 25% from existing benign nevi[9]. During the radial growth phase (RGP) the cells grow more laterally, but stay in the epidermis. Next, the tumor cells invade the upper layer of the epidermis and the underlying dermis, which is distinctive for the vertical growth phase (VGP). Thereafter the tumor will become metastatic[8] (Fig. 3).

The several steps in this process are characterized by genetic alterations. Most of the alterations are induced by environmental factors, such as UV exposure, but in 8-12% the alterations are inherited. A well-known congenital alteration in melanoma is the high amount of polymorphism in the *MC1R* gene. MC1R is a seven-transmembrane G-protein-coupled receptor expressed on melanocytes and involved in the process of pigmentation. Allelic variation of the MC1R is responsible for the 'red hair color' phenotype and the increased risk of developing a melanoma. Another congenital alteration is the loss of the cell cycle regulator *CDKN2A* in 25-40% familial melanoma cases. Loss of the *CDKN2A* tumor suppressor gene leads to the immortalization of the proliferating cells in the dysplastic and benign nevi. Besides the congenital alteration, many sporadic alterations are characterized in melanoma. *NRAS* mutations occur in 10-15% of the melanoma samples and *BRAF* mutations in 60%. The BRAF V600E mutation, which causes the constitutive activation of the BRAF kinase, is present in 80% of the *BRAF* mutations in melanoma. Mutations in the BRAF and NRAS kinases are an early event, which drive the proliferation of melanocytes in nevi. In later stage of tumor development the tumor cells undergo an epithelial-to-mesenchymal transition (EMT), which is a process in which the cells lose their epithelial characteristics and acquire more mesenchymal properties. The EMT process is a first step in the acquirement of metastatic potential. The PI3K/AKT pathway is known

to be involved in the EMT process. The tyrosine kinase AKT enhances MDM2-mediated degradation of p53, which regulates the EMT. Overactivation of AKT occurs either through amplification in 35% of the melanoma cases or through loss of the negative regulator PTEN in 37%. Metastatic melanoma has a reduced expression of E-cadherin, a protein involved in cell adhesion[8,9].

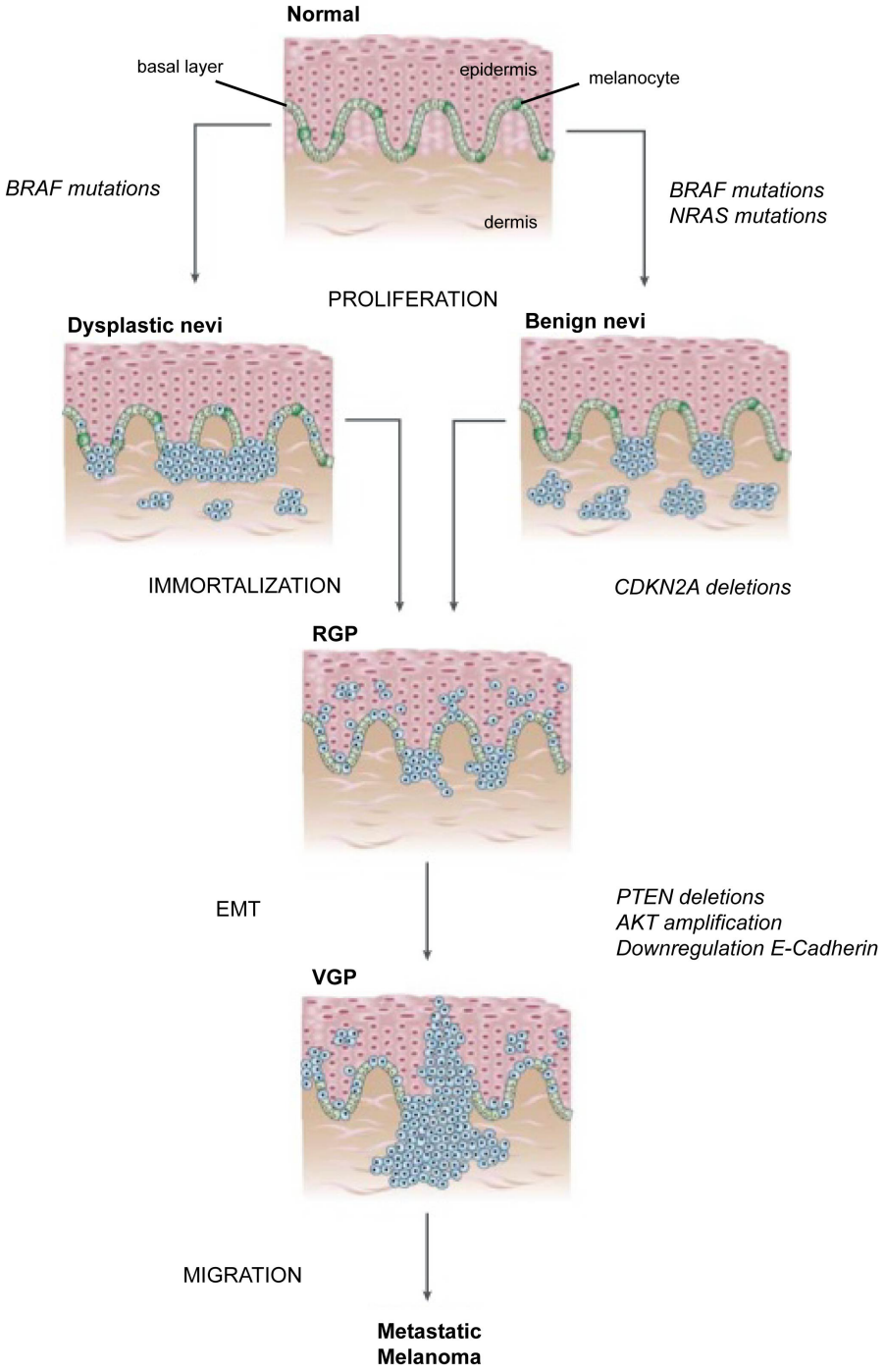


Figure 3. Stages of melanoma development Melanocytes are located in the basal layer of the epidermis and with their long dendrites they reach the keratinocytes in the superficial layers of the skin. The development of melanoma is characterized by different stages. First the amount of melanocytes increases compared to the amount of keratinocytes, which results in the formation of dysplastic or benign nevi. Most of these nevi carry BRAF or NRAF mutations, which drive the proliferation of the melanocytes. Loss of the CDKN2A cell cycle regulator causes immortalization of the melanocytes. In a next stage, the melanocytes grow laterally in the epidermis, which is called the radial growth phase (RGP). This stage is followed by the VGP whereby the cells will invade the upper layer of the epidermis and the underlying dermis driven by the epithelial-to-mesenchymal transition (EMT) process. There is evidence that loss of PTEN and AKT amplification contribute to this EMT process. The transition from RGP to VGP is assumed to be a critical in the acquirement of the metastatic character of the melanoma. In the last stage the tumor cells will migrate because of the acquirement of alterations such as downregulation of E-cadherin and which will result in the development of a metastatic melanoma. (Figure based on reference[8])

Over 90% of the early stage melanoma can be cured by surgery. However, the prognosis of late stage melanoma is poor even with chemotherapy. Therefore, many researchers continue to invest further understanding the molecular basis of melanoma in order to develop more targeted therapies. Examples are the use of tyrosine kinase inhibitors, such as the BRAF V600E specific inhibitor, vemurafenib. Although the response is good, overcoming resistance and side effects remains a big challenge[8,9].

2 Protein tyrosine phosphorylation as an important signal transduction in eukaryotic cells

The human body requires well-defined and regulated communication between cells to function properly. Cells respond to both stimuli coming from the environment and from neighboring cells. Environmental signals include light or warmth whilst neighboring cells secrete proteins and include cytokines, also called ligands. These extracellular signals are often detected by receptors located in the cell membrane, which transmit the signal inside the cell. Hereby, a cascade of intracellular signaling proteins is activated, which in the end results in a cellular response. This process of transmission of signals in a cell through a range of signaling proteins, initiated by an extracellular stimuli and resulting in a cellular response is called signal transduction[10].

Over the past 60 years it has become clear that protein tyrosine phosphorylation is an important signal transduction mechanism which results in several cellular responses including proliferation, survival, differentiation, metabolic regulation, motility, adhesion and migration. Here, a phosphate group on a tyrosine residue is the link between proteins in these signaling pathways. This process of tyrosine residue phosphorylation is a reversible posttranslational modification of proteins regulated by protein tyrosine kinases (PTK) and protein tyrosine phosphatases (PTP). PTKs transfer a phosphate group from the ATP molecule to the hydroxyl group of the tyrosine residue and thereby generally activate signaling pathways. Conversely, PTPs counteract the effect of the PTKs by removing the phosphate group again, which results mostly in the inactivation of the pathway[10,11] (Fig. 4).

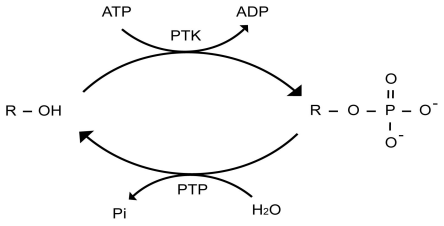


Figure 4. Regulation of protein tyrosine phosphorylation by protein tyrosine kinases and phosphatases Protein tyrosine phosphorylation is regulated by the counteracting actions of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). PTKs transfer a phosphate group from the ATP molecule to the hydroxyl group of the tyrosine residue. PTPs remove the phosphate group again by hydrolysis.

Protein tyrosine phosphorylation results not only in enzymatically activation of a protein, but can also lead to either protein stabilization or conversely mark it for proteasomal degradation. Tyrosine phosphorylation also induces protein-protein interactions or leads to protein translocation to another subcellular compartment, e.g. the nucleus where the phosphorylated protein can regulate the DNA transcription. It is now becoming clear that interruption of this tightly regulated process of protein tyrosine phosphorylation is one of the underlying causes of several human diseases like cancer[10,11].

It should be noted that in addition to tyrosine residue phosphorylation, phosphorylation of serine and threonine residues are also well-known signaling transduction mechanisms, and are regulated by protein serine/threonine kinases and phosphatases. However, since the main focus in this thesis is tyrosine phosphorylation, these mechanisms are not further discussed.

2.1 Protein tyrosine kinases

2.1.1 Classification and structure of protein tyrosine kinases

In the human genome 90 protein tyrosine kinases (PTKs) and 5 protein tyrosine pseudokinases were identified, which are highly conserved among species[12]. The 90 PTKs can be classified into 58 receptor tyrosine kinases (RTKs) and 32 non-receptor tyrosine kinases (NRTKs). According to the amino acid sequences of the kinase domains, the RTK family is further divided into 20 subfamilies and the NRTK family into 10 subfamilies[13,14] (Fig. 5). The RTKs are located in the cell membrane and contain an extracellular and an intracellular part. The extracellular part consists of several globular domains, and can include immunoglobulin (Ig)-like domains, fibronectin type III-like domains, cysteine-rich domains or EGF-like domains, which are important for ligand binding. With its transmembrane domain the RTKs are retained in the cell membrane. The intracellular part consists of a juxtamembrane domain, located just after the transmembrane domain, and a catalytic kinase domain[14-16]. In some RTKs, like the insulin receptor, the juxtamembrane domain functions as a binding site for regulatory proteins, but in others, like the PDGFR receptors, the juxtamembrane domain has an auto-inhibitory function[17]. After activation of the RTK through ligand binding the catalytic kinase domain will phosphorylate downstream signaling proteins by transferring a phosphate group from the ATP molecule.

In contrast to the RTKs, the NRTKs are localized in the cytosol, the nucleus or can be anchored to the cell membrane through modifications, such as myristoylation or palmitoylation. Myristoylation is an irreversible protein modification whereby the 14-carbon saturated fatty acid myristate is covalently attached to the N-terminal glycine residue via an amide bond[18]. Instead, palmitoylation is a reversible protein modification whereby 16-carbon saturated fatty acids are attached to specific cysteine residues through thioester linkage. Beside the catalytic kinase domain, the NRTKs contain protein-protein, protein-lipid and protein-DNA interaction domains. The most common protein-protein interaction domains found on NRTKs are the SRC homology 2 (SH2) and the SRC homology 3 (SH3) domains. The SH2 domain binds to phosphorylated tyrosine residues of other proteins and the SH3 domain binds proline-rich peptides. The TEC subfamily possesses the pleckstrin homology domain as a protein-lipid domain[14].

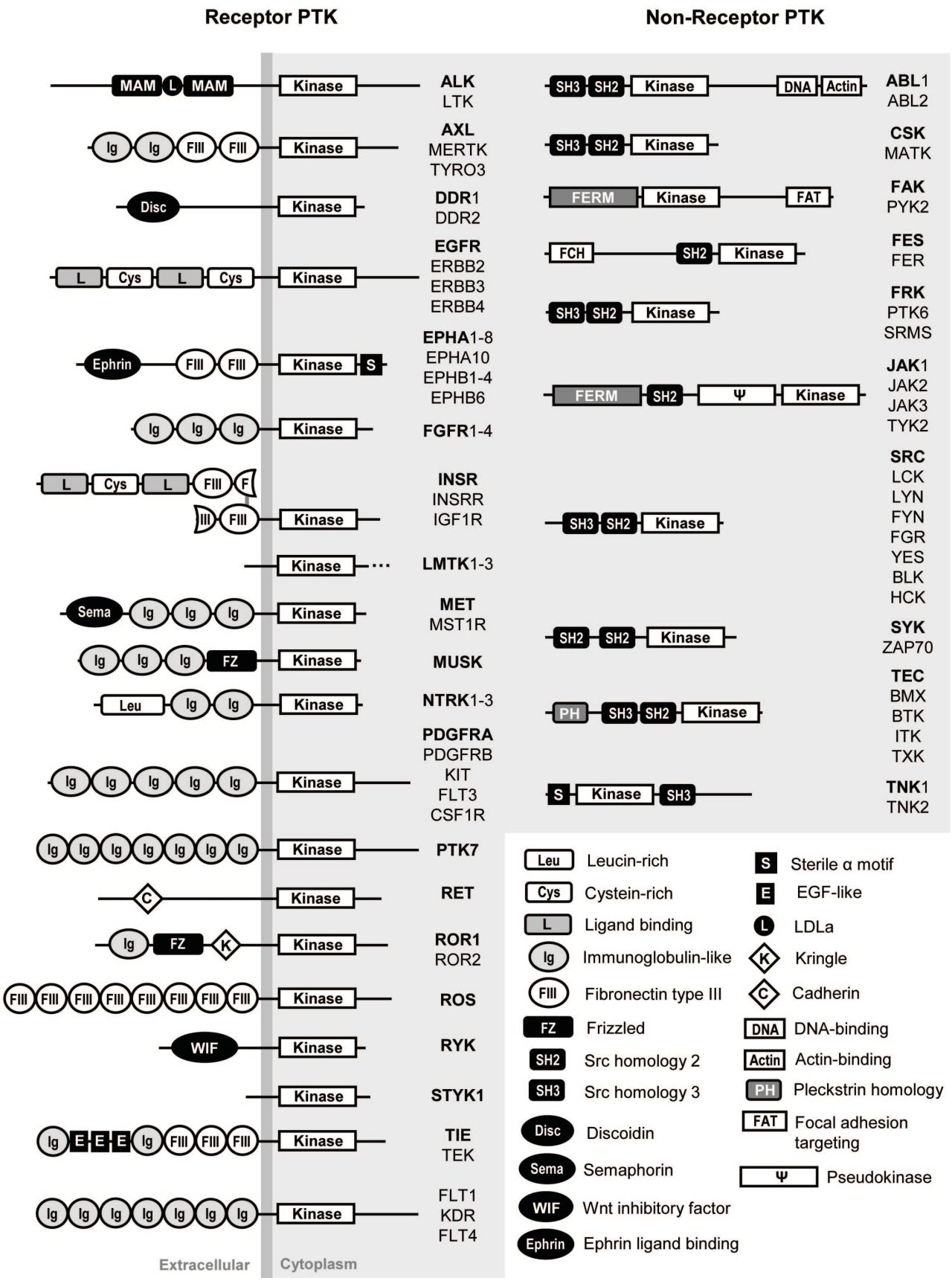


Figure 5. Classification and architecture of the protein tyrosine kinase families Schematic overview of the 90 human protein tyrosine kinases (PTKs) subdivided into receptor and non-receptor PTKs. According to their architecture the PTKs are grouped into subfamilies (indicated in bold). (Adapted from Porcu, thesis manuscript)

2.1.2 Activation of protein tyrosine kinases

2.1.2.1 Activation of receptor tyrosine kinases

With their localization in the cell membrane, the RTKs are involved in mediating the outside-in signaling. Their extracellular part can bind ligands, which will result in activation of the RTK. After activation, the intracellular part will phosphorylate and so activate downstream signaling proteins in the cytoplasm. In the inactive confirmation the RTKs can appear as a monomer (i.e. PDGFR receptors) or preformed oligomers (i.e. insulin receptor) in the cell membrane. In the absence of the ligand the RTKs are held in an inactive state by *cis*-autoinhibition of the kinase domain. There are three forms of *cis*-autoinhibition described. The first is the autoinhibition by the activation loop (e.g. as found in the insulin and FGF receptors) where a tyrosine residue in the activation loop blocks the active site for binding of ATP and protein substrates. The second form of *cis*-autoinhibition is the autoinhibition by the juxtamembrane (e.g. as found in FLT3, KIT and PDGFR receptors), where the juxtamembrane region covers the kinase domain to prevent its activation. The third form is the blocking of the active site by the C-terminal tail (e.g. as found in the TIE2 receptor). These *cis*-autoinhibition mechanisms are then overcome upon ligand binding to the extracellular part of the receptor, which results in the dimerization of receptor monomers and induces conformational changes in the dimer structure. These structural changes then facilitate tyrosine autophosphorylation in the cytoplasmatic domain. Hereby, one receptor in the dimer will phosphorylate the tyrosine residues of the other RTK. The phosphorylated tyrosine residues of the intracellular part of the RTK form docking sites for the binding of cytoplasmic proteins to pursue the downstream signaling[14,16].

2.1.2.2 Activation of non-receptor tyrosine kinases

NRTKs function in the cytoplasm downstream of the receptor tyrosine kinases. In general, NRTKs are kept in an inactive confirmation by inhibitor proteins and lipids and through *cis*-autoinhibition mechanisms. Phosphorylation of the tyrosine residues in the activation loop by RTKs or other NRTKs results in an increased kinase activity[14,15]. The mechanisms of activation can differ between NRTKs and are exemplified for the SRC, ABL1 and JAK kinases.

The human SRC family counts eight family members (SRC, LCK, FYN, YES, FGR, HCK, BLK and LYN) and all share a similar protein architecture. They contain a myristoylated amino terminus, a region with positively charged residues for the interaction with phospholipid head groups, a short region with low sequence homology, a SH3 domain, a SH2 domain, a tyrosine kinase domain and a C-terminal tail. The SRC kinases contain two important tyrosine phosphorylation residues for the regulation of their activation. The first is the Tyr-416 residue located in the activation loop and its phosphorylation is required for fully catalytic activation of the SRC kinase. The second regulatory phosphorylation site is Tyr-527 located in the C-terminal tail. Phosphorylation of Tyr-527 by the negative regulator and NRTK CSK locks the SRC kinase in a closed confirmation through binding of the phosphorylated tyrosine residue with its own SH2 domain. In addition, intramolecular interactions between the SH3 domain and the segment linking the SH2 domain with the kinase domain, called SH2-kinase linker, stabilize this closed conformation[14].

The ABL1 kinase is a cytoplasmic and nuclear NRTK that is involved in the regulation of cellular processes including cell differentiation, proliferation, adhesion and stress response. Although the structure of the ABL kinase resembles that of the SRC kinase, its activity is differently regulated. Instead of regulating the kinase activity, the C-terminal region of ABL1 is important for the protein-protein interactions and the subcellular localization of the ABL1 kinase. Instead, the SH3 domain of ABL inhibits its kinase activity by binding cellular inhibitors, such as PAG[14].

The JAK family consists of 4 members (JAK1, JAK2, JAK3 and TYK2), which share a similar architecture consisting of a FERM domain, a SH2 domain, a pseudokinase domain and a catalytically active kinase domain. With their FERM domains, the JAK kinases are bound to the intracellular part of cytokine receptors, which lack intrinsic catalytic activity. Ligand binding to the extracellular part of the cytokine receptor induces a conformational change, which brings the JAK kinases in close proximity to enable transphosphorylation and activation. The pseudokinase domain is involved in the positive and negative regulation of the kinase activity. In the absence of cytokine induction the pseudokinase domain suppresses the basal JAK activity. However, the pseudokinase domain is also required for the cytokine inducible activation of the downstream signaling. The exact mechanism behind this dual function of the pseudokinase domain is not yet clear[14,19].

2.1.2.3 Transfer of phosphate-group in the kinase domain

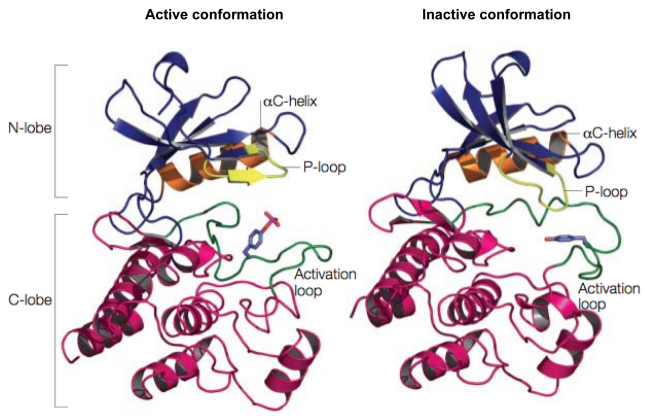


Figure 6. Structure of tyrosine kinase domain Structure of tyrosine kinase domain of SRC family kinases in active (*left*) and inactive (*right*) conformation. The N-lobe consists of 5 β-sheets and 1 α-helix and the C-lobe exists mainly out of α-helices. (Adapted from reference[22])

The architecture of the kinase domain is similar in all protein kinases and consists of small N-terminal lobe (N-lobe) and a large C-terminal lobe (C-lobe). The N-lobe contains five β-sheets and one α-helix, whereas the C-lobe mainly consists of α-helices. The deep cleft formed by the two lobes is the active site[14,20-22] (Fig. 6). The magnesium or manganese cations present in the cleft are important for the binding and coordination of the ATP molecule in the cleft. The G-loop between the β1- and β2-strands, containing three conserved glycines, covers the β- and γ-phosphates of the ATP molecule and is involved in the phosphoryl transfer and the ATP-ADP exchange. The substrate binds to the C-lobe, which brings the substrate in close proximity to the ATP molecule in order to facilitate the transfer of the phosphate-group from the ATP molecule to the substrate. In the inactive confirmation, binding of ATP and the substrate is impossible, because the activation loop is blocking the active site. Phosphorylation of the tyrosine residue in the activation loop will remove the activation loop from the active site and makes it accessible for the ATP molecule and substrates[14,21].

2.1.3 Mechanisms of protein tyrosine kinase deregulation in cancer

Deregulation of the tightly controlled tyrosine kinase activity contributes to the development of several cancer types. In this section we describe four mechanisms that are involved in the deregulation of the PTKs: fusions, mutations, increased or aberrant expression of the PTK or its ligand, and loss of negative regulators. These alterations can change the subcellular localization of the PTK or alter the signal transduction, which will promote cell proliferation and survival, and increase the invasive and metastatic character of the tumor cells[15,23].

2.1.3.1 PTK fusion proteins

Chromosomal translocations can activate a PTK via fusion with a partner protein resulting in its constitutive activation of the PTK independent of activating signals by inducing oligomerization, changing the subcellular localization or destroying an autoinhibitory domain. A well-described example of a translocation activating a PTK via the induction of oligomerization occurs in the BCR-ABL fusion protein. The fusion protein present in 95% of the chronic myeloid leukemia (CML) cases and in 25% of the adult B-ALL cases. The chromosomal translocation t(9;22)(q34;q11.2), also known as the Philadelphia chromosome, fuses the 5' part of BCR to the first intron of the NRTK ABL. The constitutive activation of the ABL kinase is caused by the oligomerization through the coiled-coiled domain in the N-terminus of BCR, through the loss of the autoinhibitory N-terminal myristoyl group and through binding of the GRB2 adaptor protein. These alterations leads ultimately to the phosphorylation of the Tyr-177 residue in BCR and concomitant activation of the RAS/MAPK and PI3K/AKT signaling pathways[24-26].

Another chromosomal translocation involving the ABL kinase is the generation of the NUP214-ABL1 fusion, which is present in 6% of the T-ALL cases. In most T-ALL patients this fusion gene is located on episomes. The episomes are formed after deletion and followed by circularization of the 9q34 region between intron 1 of ABL1 and intron 23-24 of NUP214. The formation of the episomes results in an in-frame fusion of the NUP214-ABL1 gene. The episomes are amplified during cell division and can be integrated again in the genome. In contrast to BCR-ABL fusion, the kinase activation of the NUP214-ABL1 fusion occurs because of the relocalization of ABL to the nuclear pore complex and its interaction with other nucleoporins. The wild type NUP214 protein is a nucleoporin in the nuclear pore complex, where it is involved in export of macromolecules between the nucleus and the cytoplasm. Because of the N-terminal region of the NUP214, the NUP214-ABL1 fusion protein is also localized at the nuclear pore complex. In this complex the NUP214-ABL1 fusion proteins are interacting with each other and with other nucleoporins, which results in the kinase activation of the fusion protein[27-29].

A translocation that results in the loss of a PTK autoinhibitory domain is exemplified by the FIP1L1-PDGFRα fusion found in chronic eosinofilic leukemia. In this fusion a truncated juxtamembrane domain of the RTK PDGFRα is responsible for the constitutive activation of the FIP1L1-PDGFRα fusion. As described above, the juxtamembrane domain can acts as an autoinhibition mechanism in RTKs and therefore the loss or

interruption of the juxtamembrane domain results in autophosphorylation and constitutive activation of the kinase domain[30-32].

2.1.3.2 PTK mutations

The occurrence of point mutations, small deletions and insertions in autoregulatory domains of the kinase can affect its activity. Mutations in the JAK kinase family members are often identified in hematological malignancies. JAK kinases are bound to the cytosolic domains of cytokine receptors, because of the lack of intrinsic catalytic activity of these receptors. Cytokine binding will activate the JAK kinases, which in turn will phosphorylate the STAT proteins. Phosphorylated STAT proteins will dimerize and translocate to the nucleus to regulate gene expression. The JAK2 V617F mutant is present in the majority (95%) of the polycythemia vera patients and in 50% of the essential thrombocythemia and idiopathic myelofibrosis. The V617 residue is located in the pseudokinase domain of JAK2 and maintains the activation loop in a position where it blocks the kinase domain from substrate binding. Substitution of this residue will constitutively activate the kinase, which will promote the proliferation of the malignant cells[33,34]. Mutations in the JAK member have also been identified in ALL and AML. Mutations in JAK1 have been reported in 10-20% of the T-ALL cases and mutations in JAK3 in 15% of the T-ALL cases. Compared to the high frequency of the JAK2 V617R mutant, the JAK1 and JAK3 mutations are spread all over the protein. Depending on the location of the JAK1 and JAK3 mutation, the mechanism of constitutive activation of the kinase differs. Mutations in the FERM and SH2 domain affect the cytokine receptor binding. Mutations in the kinase and pseudokinase alter ATP and substrate binding and coordination[35-39].

2.1.3.3 Increased or aberrant expression of the PTK or its ligand

The increased or aberrant expression of the PTK or its ligand is another mechanism, which enhances cellular signaling. The JAK/STAT pathway is also involved in the development of Hodgkin lymphomas, because of an amplified *JAK2* locus in 30% of the cases[39]. Another well-described example of PTK activation is the amplification of the RTK HER2/neu, which is amplified in 25-30% of the breast cancers[40].

2.1.3.4 Loss of PTK negative regulators

Besides the genetic alterations that affect the PTKs directly, loss of their negative regulators can also increase their kinase activation. The suppressors of cytokine signaling (SOCS) proteins are part of classical negative feedback loop of the JAK/STAT pathways. The STAT proteins induce the expression of the SOCS proteins, which bind the JAK proteins as pseudosubstrates, thereby inhibiting the kinase activity[41]. SOCS proteins are frequently mutated in Hodgkin lymphoma and thereby prevent the negative feedback loop resulting in the constitutive activation of the JAK2/STAT5 signal transduction pathway[39,42]. The protein tyrosine phosphatases (PTPs) are also negative regulators of PTKs by dephosphorylating their tyrosine residues. Examples are loss of the JAK/STAT negative regulators, PTPN2 and PTPRC, in T-ALL[43-45].

2.1.4 Protein tyrosine kinases as therapeutic targets

Currently chemotherapy remains the treatment of choice for most cancer types. However, although this conventional chemotherapy is effective in some cancers, the outcome is often unpredictable or just palliative. Moreover, the treated patients suffer from severe side effects including immunosuppression, anemia, hair loss, nausea and vomiting. These side effects are mostly due to mechanism of action of chemotherapeutic agents and their indiscriminant targeting of all rapid proliferating cells including not only the tumor cells but also the normal cells from the bone marrow and the gastrointestinal tract[46,47]. Therefore there remains a strong rationale for developing more targeted therapies, which target specifically cancer specific molecules responsible for driving the tumor growth and progression. In this regard, there has been a strong interest on developing inhibitors against different family members of PTKs that are frequently mutated and constitutively active in cancer and which are often essential for the proliferation and/or survival of the tumor cells[48]. To date there has been the development of two main types of PTK inhibitors: 1) small-molecule drugs and 2) monoclonal antibodies[46]. The number of these agents has been increasing steadily over the past decade and a few inhibitors have now received a US Food and Drug Administration approval as cancer treatments including imatinib mesylate which targets BCR-ABL in CML and trastuzumab which targets HER2 receptor in breast cancer[48].

Despite the promising results of the PTK inhibitors in patient treatment, there are still some challenges to overcome. The major challenge to overcome is the development of drug resistance. Several mechanisms have been described that cause drug resistance, such as the induction of secondary mutations that impede drug binding, overexpression of the oncogenic PTK or its ligand and upregulation of alternative kinase signaling pathways. This has led to the development of a new generation of inhibitors and the use of combinatory therapies as ways to circumvent this observed drug resistance[15,46,48,49]. Although the PTK inhibitors are less toxic than the conventional chemotherapy because they target the tumor cells with more specificity, still some toxic side effects have been reported due to the inhibition of PTKs in normal cells. Therefore, the search for new and more effective therapeutic targets with a higher specificity towards the oncogenic target is ongoing[15,48].

2.1.4.1 Small-molecule PTK inhibitors

The majority of small-molecule PTK inhibitors interfere with the kinase activity by interacting with the ATP-binding pocket through the formation of non-covalent hydrogen binding in a competitive manner. Other types of inhibitors include the allosteric which bind outside the ATP-binding pocket and covalent inhibitors that form irreversible and covalent bonds to the kinase active site[48]. Of the ATP-competitive inhibitors small molecule inhibitors, one of the first commercial and clinical success stories was the development of the drug imatinib mesylate (Gleevec), used for the inhibition of the oncogenic BCR-ABL fusion in CML patients. However, despite of the good response in many CML patient samples, the occurrence of imatinib resistance now presents as a significant clinical challenge[50]. The main cause of imatinib resistance is the introduction of secondary mutations in the ABL kinase domain, which interfere with the binding of imatinib to the ATP-binding pocket. A common secondary mutation in the ABL kinase domain is the T315I mutation, also

called the gatekeeper mutation, since it both interrupts the hydrogen bond to imatinib and also allosterically interferes with the drug binding[46,51]. Gene amplification and overexpression of BCR-ABL were also associated with imatinib resistance. To overcome these resistance mechanisms, a second-generation of small-molecule PTK inhibitors is now being developed. One of these agents is the dual SRC-ABL inhibitor dasatinib, which binds ABL with less strict conformational requirements than imatinib[49].

2.1.4.2 Monoclonal antibodies

Monoclonal antibodies are directed against the extracellular part of the RTKs or their ligands, which results in a diminished receptor signaling[52]. Overexpression of HER2 receptor occurs in 25% of the breast cancer patients, and these patients are treated with trastuzumab (Herceptin), a monoclonal antibody against the HER2 receptor. In combination with chemotherapy, trastuzumab increases the survival of metastatic breast cancer patients with 25% compared to patients treated with the cytotoxic chemotherapy alone[15,49,52]. Another example of monoclonal antibodies is cetuximab (Erbitux) directed against the EGFR RTK and used in combination with chemotherapy for patients with non-small-cell lung cancer, head and neck cancer and colorectal cancer[15,49,52]. However, parallel to the small-molecule PTK inhibitors, overcoming drug resistance and increasing the sensitivity are the major challenges in the development of new monoclonal antibodies.

2.2 Protein tyrosine phosphatases

2.2.1 Classification and structure of protein tyrosine phosphatases

Protein tyrosine phosphatases (PTPs) dephosphorylate the tyrosine residues of substrates and thereby form the counterbalance of the PTKs, which phosphorylate the tyrosine residues of their substrates. In total there are 107 PTPs, but some of them are catalytically inactive or dephosphorylate mRNA or inositol phospholipids. This reduces the number to 81 PTPs catalytically active PTPs, which act to dephosphorylate phospho-tyrosine residues. This number is comparable with the 85 catalytically active PTKs, highlighting the important balance between PTKs and PTPs. According to the amino acid sequence and their catalytic domain the PTPs can be grouped into three classes of Cys-based PTPs and one class of Asp-based PTPs[53,54] (Fig. 7). In contrast to the three cysteine-based PTPs classes, in the class of Asp-based PTPs the aspartic acid residue has a central role in the catalytic mechanism[54,55]. The **class I Cys-based PTPs** can be subdivided into 38 “classical” PTPs, which are strictly tyrosine specific, and 61 dual specificity phosphatases (DSPs), which dephosphorylate Tyr and Ser/Thr residues, phosphatidyl inositol phosphates and mRNA[53,54,56]. The “classical” PTPs are further divided in 21 receptor like PTPs (PTPR) and 17 non-receptor PTPs (PTPN). Through their location in the transmembrane, the RPTPs can bind a high diversity of ligands with their diverse extracellular parts built of immunoglobulin (Ig)-like, fibronectin type III (FNIII) like, mephrin/A5/RPTPμ (MAM), carbonic anhydrase-like and/or cysteine-rich domains. The identity and function of these ligands are not completely known. The Ig-like, FNIII and MAM regions are also found in cell adhesion molecules, predicting a role of PTPR in cell-cell contact[57]. Out of the 21 PTPR, 12 contain two PTP domains. With the exception of PTPRA, only the first domain is catalytically active. The second PTP domain

is involved in regulation of the catalytic activity of the first domain, in protein-protein interaction during dimerization and in substrate binding. The PTPNs are located in the cytoplasm and consist of one catalytically active PTP domain, the SH2 or FERM binding domains and regulatory domains, which control the phosphatase activity and subcellular localization[53,55,56,58].

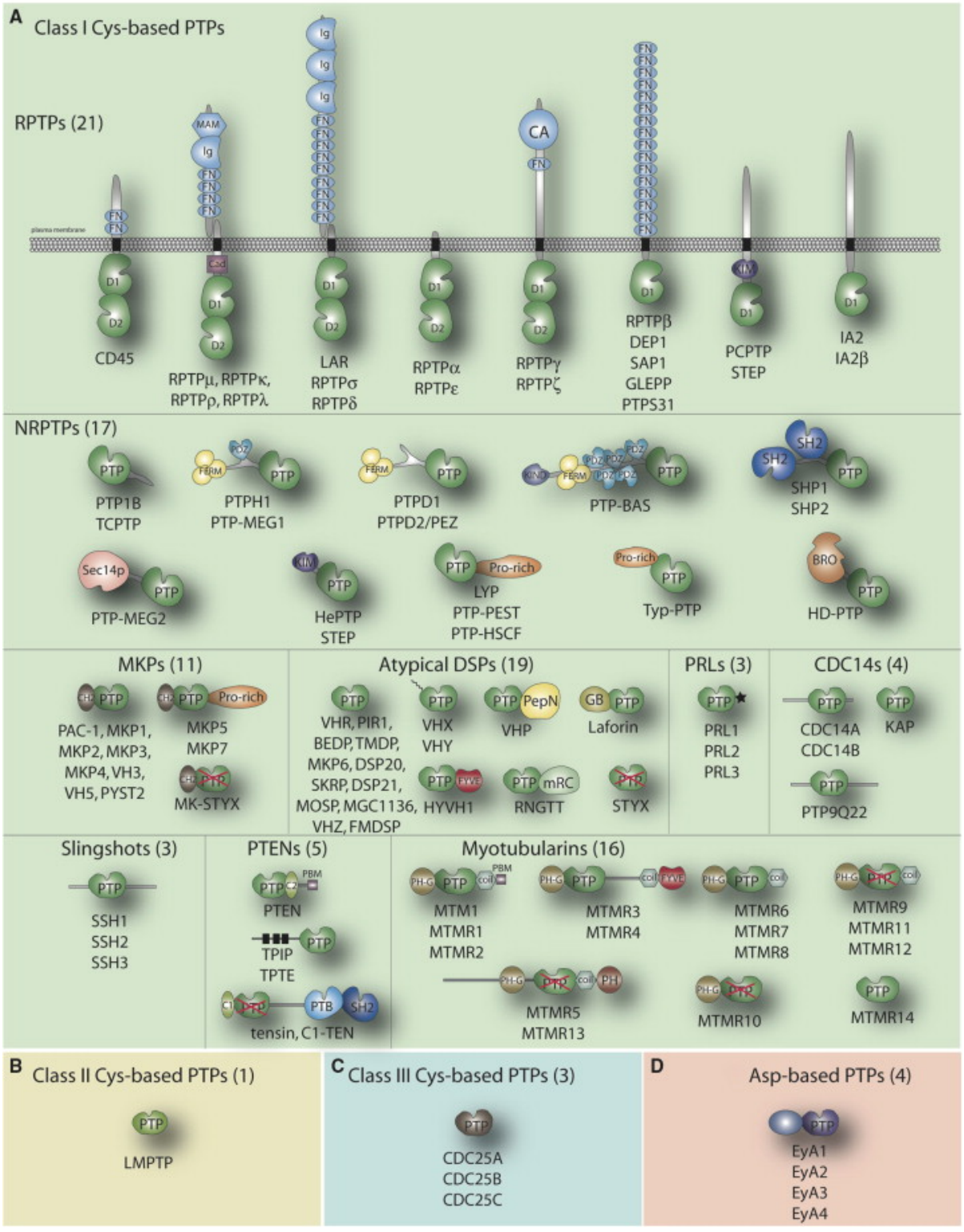


Figure 7. Classification and architecture of the protein tyrosine phosphates Schematic overview of the human protein tyrosine phosphates, which are grouped into four classes according to their amino acid sequence and their catalytic domain. Used abbreviations; Coil: coiled-coil domain, GB: glycogen binding, mRC: mRNA capping, PBM: PZD binding motif, pepN: N-terminal peptidase-like, PH-G: pleckstrin homology-"GRAM" domain, Pro-rich: proline rich, Sec14: Sec14p homology. A red cross over a PTP domain indicates a catalytically inactive domain. (Adapted from reference[54])

In contrast to the “classical” PTP, the DSPs have less sequence similarity, but they use the same catalytic mechanism with a key role for the cysteine residue. Because of alternative active site conformation, the DSPs are able to dephosphorylate a broader range of substrates. The DSP can be further divided in 7 subgroups: MAPK phosphatases (MKPs), atypical DSPs, Slingshots, PRLs, CDC14s, PTENs and Myotubularins. The **class II Cys-based PTPs** contains the Low Molecular Weight (LMW) PTPs, which has very low sequence homology compared to the other PTPs, except from active site motif[54,58]. The rhodanese-derived PTPs belong to the **class III Cys-based PTPs**. They regulate the cell cycle through dephosphorylation of the CDKs proteins. In contrast to the Cys-based PTP, an aspartic acid is required in the catalytic mechanism of the **class IV Asp-based PTPs**. To this group belong the EyA phosphatases[53,54].

2.2.2 Regulation of protein tyrosine phosphatases and mechanism of dephosphorylation

The activity of PTPs is tightly regulated and are not constitutively active housekeeping enzymes as initially thought. These different regulatory mechanisms include controlling their ability to dimerize, their phosphorylation status, and their oxidative status. The ability of PTPs to **dimerize** is a known regulatory mechanism in many RPTPs, such as PTPRA, PTPRC, PTPRS. When RPTPs are dimerized the helix-turn-helix wedge loop from one PTP domain blocks the PTP domain of its partner, which results in the inhibition of the catalytic activity. This dimerization may be induced or interrupted by **ligand** binding. This is in sharp contrast with regulation of PTKs, where ligand-induced dimerization results in kinase activity. The first evidence that dimerization could regulate PTP activity was found for RPTP using a chimeric protein in T-cells. The chimera contained the extracellular part of the EGFR kinase and the intracellular part of PTPRC. Dimerization of the chimera was induced after addition of the EGF ligand, which resulted in a reduced T-cell response due to inactivation of the chimera. A second mechanism to regulate PTP activity is via **phosphorylation**. An example includes the regulation of PTPRA activity, which is activated after phosphorylation of the two serine residues in the juxtamembrane domain. Here, phosphorylation of the two serine residues results in disruption of the inactive dimer confirmation, since the serine residues are closely located to the helix-turn-helix wedge loop. Finally, the phosphatase activity of the “classical” PTPs including, cdc25c, PTEN and LMW-PTPs are negatively regulated by **oxidation**. The cysteine residue in the active site of the PTPs is highly sensitive to oxidation because of its thiolate state (S-). Oxidation of this nucleophilic cysteine results in a conformational change that abolishes the substrate binding. The oxidation is mainly caused by the formation of reactive oxygen species (ROS) in the cell, but also UV-radiation, cell adherence and density can stimulate oxidation[55,59,60].

Once active, the PTPs can dephosphorylate their substrates through hydrolysis. Here we describe the catalytic mechanism of Cys-based PTPs (Fig. 8). All Cys-based PTPs contain a phosphate binding loop or P-loop with the PTP signature motif (H/V)C$(X)_5$R(S/T) in their active site. In a first step, the thiolate side chain of the cysteine residue in the P-loop functions as a nucleophile by the donation of an electron pair to accept the phosphoryl group from the substrate. The arginine residue in the P-loop forms two hydrogen bonds with the phosphoryl group through its guanidinium group to stabilize the substrate binding. The WPD loop closes around phospho-tyrosine. The aspartic acid residue in the WPD loop behaves as an acid by protonating the tyrosyl group of the substrate. This will result in the demerger of the substrate. In a second step, the release

of the phosphate occurs by hydrolysis of the cysteinyl-phosphate intermediate. Hydrolysis is mediated by the glutamine of the Q-loop, which coordinates the nucleophilic water molecule, and the aspartic acid of the WPD-loop, which acts now as a general base[55,58,61].

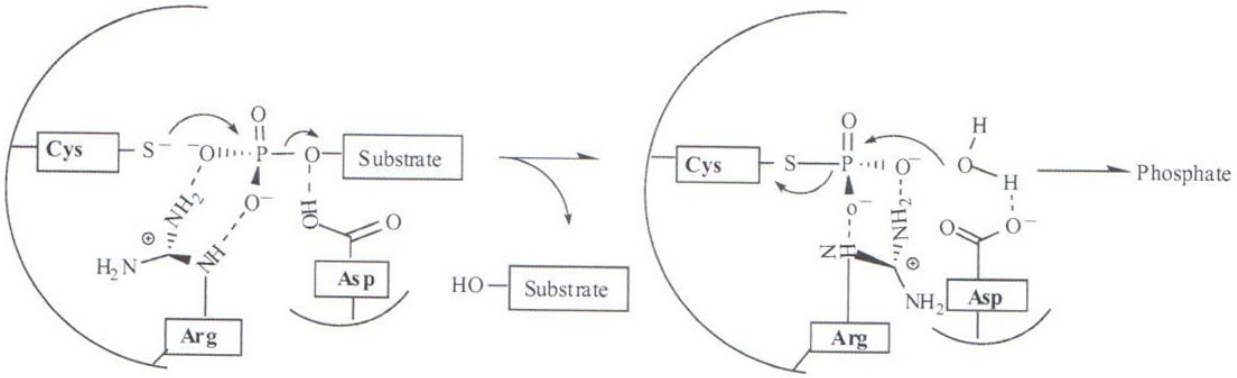


Figure 8. Catalytic mechanism of Cys-based protein tyrosine phosphatases The catalytic mechanism of Cys-based PTPs occur in two steps. In the first step the thiolate side chain of the cysteine residue in the P-loop donates an electron pair to the phosphoryl group of the substrate. For stabilization of the substrate binding the arginine residue in the P-loop forms two hydrogen bonds with the phosphoryl group. The aspartic acid residue in the WPD loop protonate the tyrosyl group of the substrate. This results in the release of the substrate. In a second step the phosphate is released through hydrolysis. Therefore, the glutamine of the Q-loop coordinates the nucleophilic water molecule and the aspartatic acid of the WPD-loop behaves now as a base. (Adapted from reference[61])

2.2.3 Protein tyrosine phosphatases in cancer

PTKs together with PTPs control the balance of tyrosine phosphorylation, which is important for downstream signal transduction and regulation of several cellular processes such as proliferation, survival, differentiation, adhesion and migration. Disruption of this tightly regulated balance contributes to the development of human diseases, such as cancer. As described above, deregulation of this balance can occurs on one hand through disturbance of the PTK activity, which promotes tumor development. On the other hand, more insight has been acquired in the contribution of altered PTP activity in the development of cancer. Here, we describe some examples of genetic and epigenetic alterations of PTP genes in cancer.

2.2.3.1 Genetic alterations

Genetic alterations affecting PTP genes include deletions, loss of heterozygosity (LOH), amplifications and mutations[56,62,63]. The most commonly lost PTP in many cancer types through genetic alterations is *PTEN*. The DSP PTEN inhibits cell proliferation and survival by inactivating the PI3K pathway. In general, *PTEN* is mutated in 15% of cancers, with a higher mutation rate in glioblastoma multiforme (GBM) (30-40%) and endometrial carcinoma (50%). LOH is also found at high frequency for many cancers including 40-55% in endometrial carcinoma, 50-70% in GBM, 31% in breast cancer and 47% in lung tumors[64,65]. In addition to *PTEN*, several other PTP family members have also been found to frequently deleted or amplified in human cancers. For example, *PTPN2* is deleted in 6% of the T-ALL cases[43] and *PTPRD* is deleted in several types of cancer such as GBM and neuroblastoma[66-70].

Inactivation of the PTPs by point mutations can also contribute to the development of cancer. The PTPRT phosphatase, for example, is mutated in 17% of the lung and gastric cancers and in 26% of the colorectal cancer[71,72]. The point mutations in the catalytic domains of PTPRT cause a reduced phosphatase activity[62,71]. As mentioned above, the members of the RPTP have already described roles in cell adhesion. Hence mutations in RPTP not only interfere with their phosphatase activity, but mutations in these RPTPs also promote tumor progression by affecting their adhesion function. Crystal structures of PTPRM phosphatase showed that mutations can reduce the adhesiveness by disrupting the homophilic interactions or affecting the protein folding or processing[73].

It should be noted that although the majority of PTPs behave as tumor suppressors as a function of their ability to inactivate the PTK signaling pathways, activating point mutations and gene amplification could contribute to the oncogenic character of some PTPs. For instance, the *PTPN1* phosphatase gene is amplified in epithelial cancers, *PTPN7* in leukemia and myelomas and *PTP4A3* in metastatic colorectal cancers[62]. The *PTPN11* phosphatase gene is mutated in 35% of the patients with juvenile myelomonocytic leukemia. However in this case PTPN11, which normally regulates activation of the RAS pathway by dephosphorylating the negative regulators, CSK and the RAS-GTPase activating protein (RASGAP), the acquired mutations enhance the PTPN11 affinity for phospho-tyrosine peptides to increase the PTP activity resulting in an enhanced RAS signaling[74-77].

2.2.3.2 Epigenetic alterations

The most common epigenetic alteration resulting in gene silencing is the DNA methylation of the CpG islands[62]. Reduction in gene expression because of DNA methylation was observed for PTPRG in 27% of the cutaneous T-cell lymphoma[78], for PTPRO in 82% of the chronic lymphocytic leukemia[79] and for PTPN13 in 50% of the hepatocellular carcinoma[80].

2.2.4 Protein tyrosine phosphatases as therapeutic targets

As well as PTKs, PTPs are also gaining interest as therapeutic targets because of their involvement in several diseases. Nevertheless many compounds targeting oncogenic PTP have not yet made it to the clinics due to a number of challenges in this research field. One of the major problems is specific targeting the active site of single PTPs because of the high level of homology in catalytic domains between the different PTP family members. In addition, potential drugs often contain oxidizing agents, which leads to the oxidation of the PTPs[56,62]. Yet, the industry has been able to develop a potent, specific and reversible inhibitor for PTP1B, which is the nonhydrolyzable pTyr-mimetics. One disadvantage of this compound is the high charge, which reduced its oral bioavailability[56,81]. The development of allosteric inhibitors would overcome these problems, because these compounds bind outside the active site inducing a conformational change, which will result in the inhibition of the phosphatase activity[56,62]. An alternative strategy to small molecule inhibitors is the use of antisense-based therapeutics, which have the advantage of higher specificity due to being sequenced based. In this instance, an antisense-based therapeutic targeting PTP1B

has shown high efficacy in clinical trials for type II diabetes[56,81]. However, before more therapeutic agents can be designed, it is imperative that a greater understanding occurs for the functional role of PTPs in cell biology and their contribution to the development of diseases. A single PTP can regulate several signaling pathways in a cell or contain multiple effects. A PTP can stimulate or inhibit signaling pathways depending on its cellular context. Several PTPs can also work together in the regulation of a signaling pathway. Therefore, understanding the signaling pathways controlled by tumor suppressive PTPs will help to identify possible targets for therapy. For example, loss of PTPN2 results in an increased activation of the NUP214-ABL1 kinase and the JAK/STAT pathway, which are proven to be therapeutic targets in the treatment of ALL[43,44].

2.2.5 Protein tyrosine phosphatase receptor delta

2.2.5.1 Structure and regulation

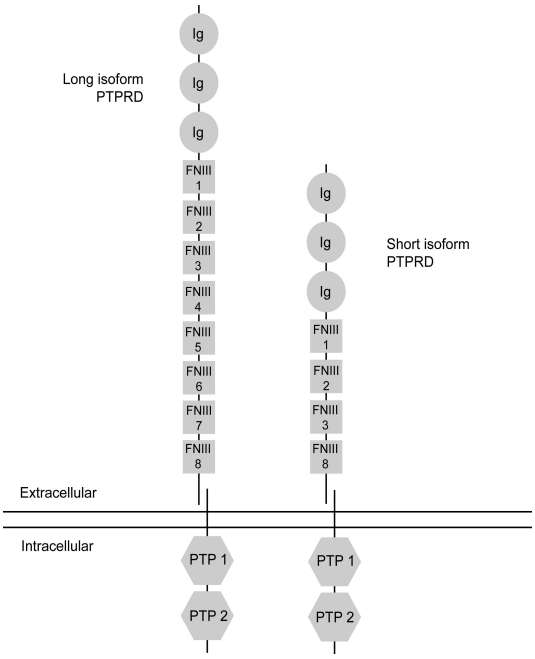


Figure 9. Schematic representation of the PTPRD isoforms The long and short isoforms of PTPRD are located in the cell membrane and differ in the amount of fibronectin type III (FNIII) like domain of their extracellular part. Both isoforms have three Ig-like (Ig) domains extracellular and contain intracellular two phosphatase domains (PTP) of which only the first one is catalytically active.

The Protein Tyrosine Phosphatase Receptor Delta (PTPRD) protein is a member of the leukocyte common antigen-related (LAR) family of proteins. The *PTPRD* gene is located on the p-arm of the human chromosome 9 (9p23-24.1). The extracellular part of PTPRD exists of three Ig-like domains and four to eight FNIII domains, depending on the isoform. The long isoform contains eight FNIII domains, whereas the short isoform lacks FNIII domain 5 to 7. The short isoform is mainly expressed in the brain[82,83]. Further, several transcripts of the short isoform have been identified lacking a few amino acids in the extracellular domain. Probably the variation in the extracellular part is involved in the binding of different ligands[82]. Segregated from the extracellular part by a transmembrane domain, the intracellular part contains two phosphatase domains of which only the first one is catalytically active (Fig. 9). The identity and the functional role of the ligands of PTPRD are not yet known. In neurons PTPRD is involved in cell-cell adhesion by the heterophilic binding of netrin-G-ligand and by the formation of homophilic bindings with the PTPRD proteins expressed on the neighboring cells[84-87].

The regulation of the PTPRD phosphatase activity is not yet understood. It is not clear whether the phosphatase activity is inhibited by ligand-binding induced dimerization of the receptor as shown for PTPRS and PTPRA[55,59,60]. What is known is that LAR family members undergo a posttranslational proteolysis, similar to the proteolytic cleavage of the NOTCH receptor. As such, the PTPRD protein is cleaved in the Golgi by a furine-like protease and both parts are held together by a non-covalent interaction[82,84]. Three

potential furin-like cleavage sites (RXXR) are located in the extracellular part of PTPRD close to the transmembrane domain, but to date it is not yet known which furin-like protease is involved in the cleavage process. Once PTPRD has travelled to the cell membrane, another proteolytic cleavage event occurs on the extracellular side adjacent to the transmembrane resulting in the release of the extracellular part. Next, the remaining 85 kDa transmembrane protein is cleaved on the intracellular side resulting in the release of a 75 kDa intracellular part[82,84,88] (Fig. 10). These extracellular and intracellular cleavage events are likely to be processed by an ADAM metalloprotease and γ-secretase complex respectively, because cleavage of the structural related PTPRM and PTPRK proteins occurs by the same protease[89,90].

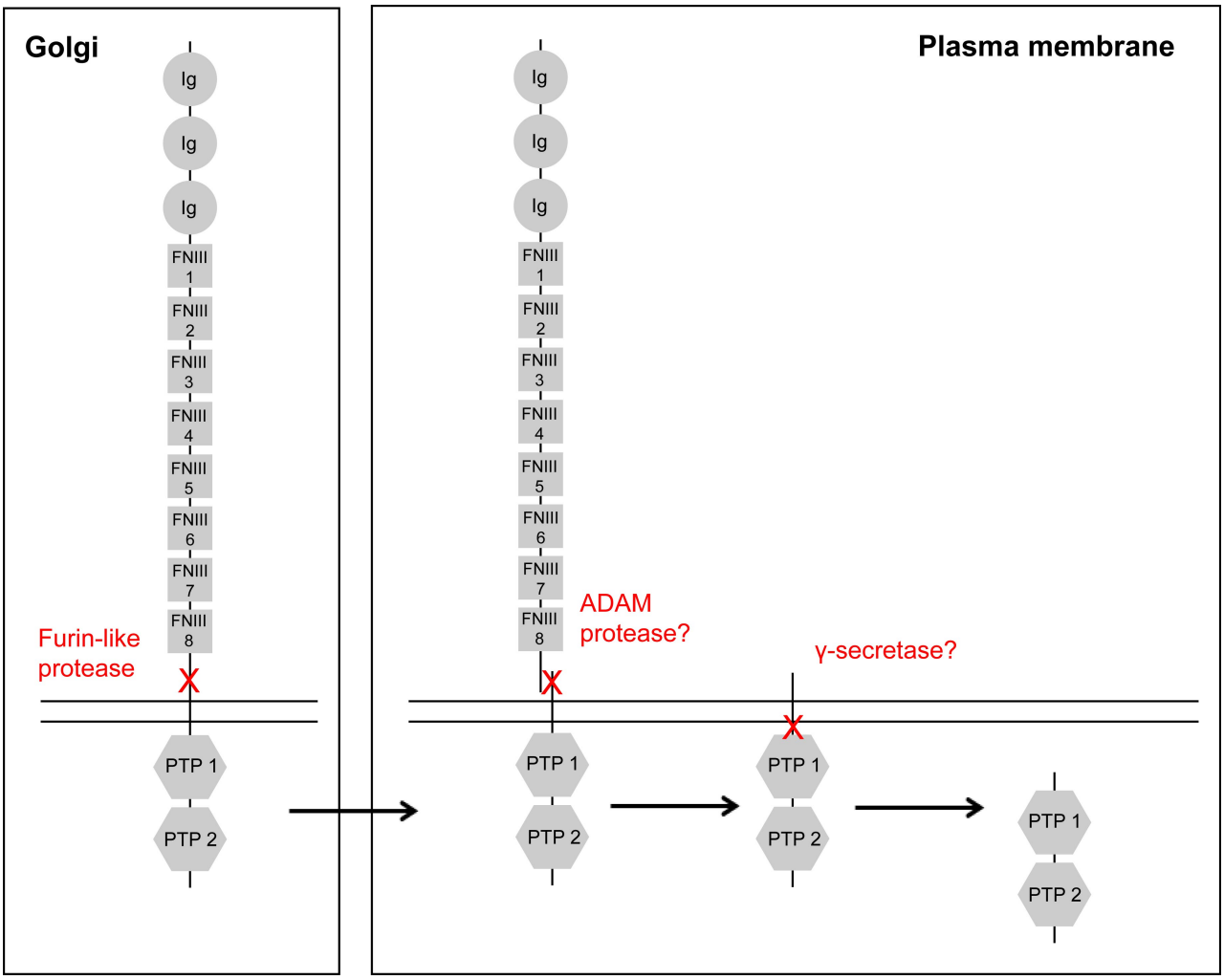


Figure 10. Proteolytic cleavage of PTPRD After cleavage in the Golgi by a furin-like protease both parts are held together by a non-covalent interaction. The PTPRD protein is transported to the cell membrane where it is cleaved in its extracellular part near by the transmembrane domain. Thereafter the protein is cleaved intracellular which results in the release of the intracellular part. The exact proteases involved in this cleavage process are not yet identified, but the ADAM proteases and the γ-secretase are potential candidates.

2.2.5.2 Biological role of PTPRD in neurons

The functional role of PTPRD in neural development and physiology has been extensively studied due to its high expression in the brain. The PTPRD protein promotes synapse formation between axons and dendrites. Present on the presynapse, PTPRD binds with its extracellular part to postsynaptic proteins, including NGL-3, IL1RAPL1 and the Slitrks proteins. These interactions induce pre- and postsynaptic differentiation,

which results in the formation of an excitatory synapse. Only binding of PTPRD with Slitrk-3 will induce the formation of an inhibitory synapse. Presynaptic differentiation is mediated by the binding of Liprin-α or MIM with the second phosphatase domain of PTPRD[87,91-93]. Homophilic interactions between PTPRD proteins promote neuronal adhesion and neurite growth in chicken neurons[86]. The long isoform of PTPRD has been found to promote neuronal adhesion, whereas the short isoform promotes neurite growth, with this latter function dependent on the phosphatase activity[94-96].

The functional importance of PTPRD in brain is also evident from the observed neurological problems in the *Ptprd*$^{-/-}$ mice. The *Ptprd*$^{-/-}$ mice exhibit motorial dysfunction, spatial learning impairment and enhanced long-term potentiation in the hippocampus. Because of their impaired spatial learning it is necessary to place the food on the floor of their cages to avoid lethality because of reduced food intake[97,98]. The possible role of PTPRD dysfunction in human neuronal disorders has not yet been demonstrated. However, in a genome-wide association study the *PTPRD* locus was associated with the restless leg syndrome[99,100].

2.2.5.3 Role of PTPRD in cancer

Genetic alterations in the *PTPRD* gene, including mutations, deletions and promoter methylation, have been described in several cancer types. The *PTPRD* gene is deleted in 12% of neuroblastomas[66,68], in 37% of metastatic cutaneous squamous cell carcinomas[101,102], in 40% of GBM[69,70], in 20% of head and neck cancers cell lines, in 11% of lung cancer cell lines[103] and in 4% of primary lung cancer samples. Mutations in the *PTPRD* gene are observed in 37% of metastatic cutaneous squamous cell carcinomas[101,102], in 6% of GBM, in 13% of head and neck cancers[70], in 6-9% of lung cancers[103-106] and in 12% of melanomas[69,107,108]. Reduced PTPRD expression because of promoter methylation occurs in 37% of GBM[70] and in high-grade breast cancers and colorectal cancers[109,110].

Functional studies have established that PTPRD has a growth suppressive role in human cancer cells. Reconstitution of PTPRD in GBM, melanoma and neuroblastoma cell lines suppresses cell proliferation and increases apoptosis, whereas shRNA-mediated knockdown of PTPRD results in an increased cell growth. The PTPRD mutants are less potent in suppressing cell growth compared to wild type in these cancer cell lines[69,70,111]. The regulation of the cell proliferation by PTPRD is ascribed to its dephosphorylation of the STAT3 transcription factor, which is involved in many proliferation pathways[70,109]. The AURKA kinase is another substrate of PTPRD identified in neuroblastoma cell lines. The AURKA kinase is a regulator of the cell cycle, which is involved in the formation of microtubule and stabilization of the MYCN oncogene. Dephosphorylation of AURKA by PTPRD results in the destabilization of the kinase and consequently of the MYCN oncogene, which is frequently amplified in neuroblastoma[111]. In addition to the regulation of cell proliferation, PTPRD can control cell migration. In colorectal cell lines, siRNA-mediated knockdown of PTPRD promotes cell migration independent of the catalytic activity. The β-catenin/TCF signaling and the CD44 expression mediate the cell migration suppression by PTPRD. The cytoskeletal regulators, Liprin-α and MIM, are assumed to be involved in the regulation of migration, because they are able to bind the second phosphatase domain[92,93,112]. Recently it was shown that heterozygous loss of *Ptprd* cooperates with *Cdkn2a* deletion in tumor formation in a glioblastoma mouse model. The *Ptprd*$^{+/-}$*p16*$^{-/-}$ mice had worse

survival outcome and developed a bigger tumor with an increased pSTAT3 level compared to the *Ptprd*$^{-/-}$*p16*$^{-/-}$ and *Ptprd*$^{+/-}$*p16*$^{+/+}$ mice. This increase in tumor volume was not caused by increased cell proliferation, but was due to the increase in protumor macrophages[113]. Considering the observed (epi-)genetic alteration in *PTPRD* together with the functional data, *PTPRD* seems to be a candidate tumor suppressor gene in the development of several cancer types.

2.2.6 Protein tyrosine phosphatase, non-receptor type 2

2.2.6.1 Structure and isoforms

PTPN2 is an intracellular, non-receptor protein tyrosine phosphatase, located on the p-arm of the human chromosome 18 (18p11.2-11.3). Although PTPN2 initially was isolated from a human T-cell cDNA library, and therefore also named T-cell protein tyrosine phosphatase (TC-PTP), the phosphatase is ubiquitously expressed in embryonic and adult tissues with a predominant expression in the hematopoietic tissues[114-117]. Alternative splicing at exon 9 results in the expression of a 45 kDa (TC45) and a 48 kDa (TC48) isoform, which differ in their C-terminus (Fig. 11). The TC45 contains at its C-terminus a nuclear localization signal (NLS), which is responsible for its localization in the nucleus. The nuclear pore complexes import TC45 in the nucleus, but stress induces the exit by passive diffusion. The TC48 is located in the endoplasmic reticulum, because the NLS sequence is masked through a hydrophobic sequence at the C-terminus, which prevents it from nuclear import. Because of their difference in subcellular localization, TC45 and TC48 have different substrates[117-120]. For example, TC48 dephosphorylates BCR-ABL and RTKs such as EGFR, the insulin receptor and the MET receptor. The TC45 isoform dephosphorylates proteins present in the nucleus, such as STAT proteins, but because TC45 is also present in the cytoplasm it also dephosphorylates RTKs, such as the insulin receptor, EGFR, PDGFR and VEGFR[116,117].

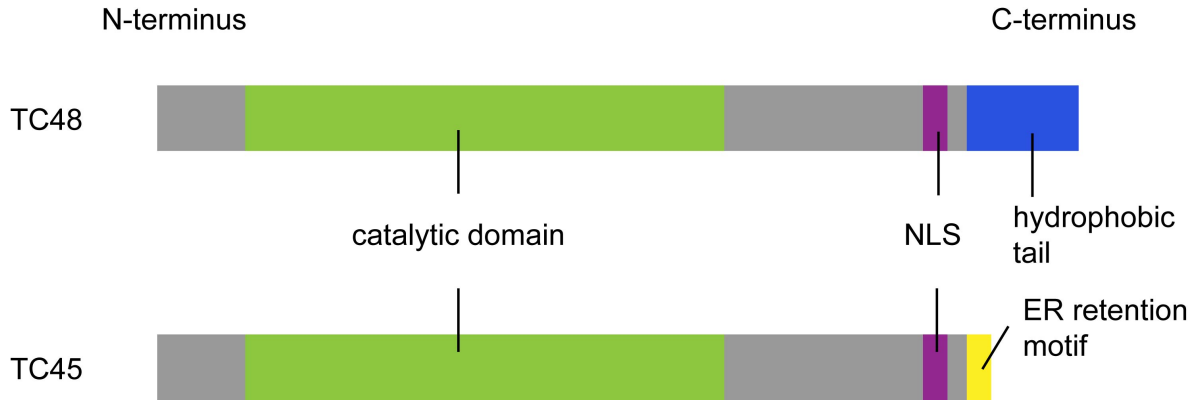


Figure 11. Structure of PTPN2 Schematic representation of the two PTPN2 isoforms, TC45 and TC48, which differ in their carboxyl terminus due to alternative splicing at exon 9. The 45 kDa isoform TC45 consist of a nuclear localization signal (NLS), whereas the 48 kDa isoform TC48 contains a hydrophobic tail which masks the NLS sequence.

2.2.6.2 Role of PTPN2 in immune response and diseases

The study of PTPN2 loss in *Ptpn2* knockout models showed evidence of the role of PTPN2 in immune response and its link with human immunological diseases. Because of the high expression in hematopoietic cells, it is not surprising that the *Ptpn2* knockout mice have defects in several hematopoietic lineages. They suffer from severe anemia and systemic inflammation, characterized by increased cytokines levels, which cause their death 3-5 weeks after birth[117,121]. The involvement of PTPN2 in the T-cell response was studied in more detail in the *Lck-Cre; Ptpn2*$^{fl/fl}$ mice. The T-cell receptor (TCR) signaling was elevated and they also had increased levels of proinflammatory cytokines. The $CD8^+$ T-cell response was enhanced after stimulation with low affinity antigens that otherwise could not induce T-cell activation and proliferation[122]. The genome wide association study point out an association between the *PTPN2* locus and the susceptibility to three autoimmune diseases (Crohn's disease, rheumatoid arthritis and type 1 diabetes) because of the identification of single nucleotide polymorphisms (SNPs). The *Ptpn2*$^{-/-}$ mice have many immune phenotypic characteristics also seen in the human Crohn's disease. Examples are diarrhea, high expression of the IFN-γ, TNF-α and IL-12 cytokines and increased sensitivity to intestinal bacteria[117]. The contribution of PTPN2 in type I diabetes can be ascribed to its regulation of the insulin receptor[117,123-128]. The fragile bones of the *Ptpn2*$^{-/-}$ mice and the elevated cytokine levels suggest a role of PTPN2 in the development of rheumatoid arthritis, although the exact mechanism is not understood[117].

2.2.6.3 Role of PTPN2 in T-cell acute lymphoblastic leukemia

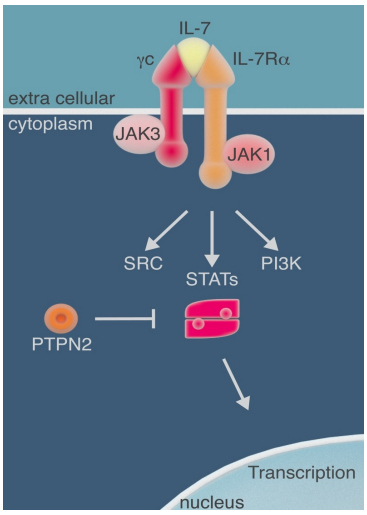


Figure 12. The IL-7 receptor pathway The non-receptor protein tyrosine phosphatase, PTPN2, is a negative regulator of the JAK/STAT pathway downstream of the IL-7 receptor in T-cells.

Besides its role in immune responses and diseases, loss of PTPN2 in hematopoietic cell also contributes to the development of hematological malignancies. Sequence analysis revealed PTPN2 inactivation in T-cell non-hodgkin lymphoma at low frequency[129]. Deletions of *PTPN2* are identified in 6% of the T-ALL cases and are specifically associated with ectopic expression of the homeobox transcription factor TLX1 in 33% of these cases. Loss of PTPN2 causes increased JAK/STAT phosphorylation after stimulation of the IL-2 and IL-7 receptors in leukemic cells, but was not sufficient to transform these cells[43,44]. The identification of JAK1 and JAK3 kinases as substrates of PTPN2 was shown earlier in substrate trapping experiments[130]. These data have shown that PTPN2 is a negative regulator of the IL-7 receptor pathway, which is an important proliferative pathway in T-cells[131] (Fig. 12). Overexpression of oncogenic kinases, such as NUP214-ABL1 and JAK1 mutants, together with knockdown of PTPN2 accelerate the transformation of the leukemic cells. Co-precipitation of the NUP214-ABL1 fusion with the catalytic inactive PTPN2 mutant D182A indicates NUP214-ABL1 as a direct substrate of PTPN2. Loss of PTPN2 prolonged the survival of a NUP214-ABL1 expressing leukemic cell line after treatment with imatinib. Knockdown of PTPN2 in leukemic cell lines expressing a JAK1 mutant are also less sensitive to JAK inhibitors[43,44].

CHAPTER II – RATIONALE AND AIMS

Protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) are enzymes involved in controlling tyrosine phosphorylation and dephosphorylation respectively. As such, they play integral roles in regulating the activity of almost all signal transduction pathways. Deregulation of the tightly controlled balance between tyrosine phosphorylation and dephosphorylation is known to be an important contributing factor in cancer development. The mechanisms resulting in constitutive activation of oncogenic PTKs have been intensively studied during the last few decades. There is now accumulating evidence that PTPs also contribute in the development of cancer, either as a tumor suppressor or as an oncogene.

It has been established that the constitutive activation of oncogenic PTKs results in the transformation of cytokine dependent cultured cells, which makes them an interesting model to study the transforming capacity of newly identified PTK mutants and to screen for drug compounds targeting the oncogenic PTKs. Our first aim was to use this transforming capacity of oncogenic PTKs in a cytokine dependent cell line to develop a screening system for the selection of functional RNAi molecules.

In acute lymphoblastic leukemia, there are numerous activated oncogenic PTKs identified including JAK and ABL1. However, little is known about which PTPs are deregulated during ALL progression. Since the PTPRD phosphatase is described to be lost in several types of solid cancer, including melanoma, our second aim was to determine whether genetic alterations of PTPRD also contribute to the pathogenesis of ALL. Next to our study in ALL, we also investigated the role of PTPRD as a tumor suppressor in melanoma to gain more insights in its functional role. Previously, the PTPN2 phosphatase is identified as a tumor suppressor in ALL, which negatively regulates the JAK/STAT pathway. Our third aim was to identify other signaling pathways controlled by PTPN2 in the development of T-ALL.

In more detail we aimed to:

1. Develop a siRNA and shRNA screening system based on a kinase fusion protein

Although RNAi design algorithms have improved over the last years, it is still a challenge to identify effective and specific siRNAs and shRNAs, and this is sometimes further complicated by the difficulty of transfection or transduction of the cells of interest. It was my first aim to develop an easy cell-based system that would allow the identification of effective siRNA and shRNA molecules in one specific cell line that is easy to transduce and to transfect.

2. Study a possible tumor suppressive role of PTPRD in ALL and melanoma

The tyrosine phosphatase gene *PTPRD* is described to be a candidate tumor suppressor gene in several solid cancers, because it is frequently inactivated by genetic and epigenetic alterations. Unfortunately, gene expression and functional data are lacking to prove this assumption and the contribution of PTPRD in the pathogenesis of ALL was not investigated.

Therefore, the aim of this part is to:

A. Study the role of PTPRD as a tumor suppressor in ALL

To determine the frequency of genetic alteration in ALL, I analyzed copy number variations (CNV) and mutations in ALL patient samples and cell lines. The correlation between CNV and the expression levels were defined after analysis of gene expression profiles. The role of PTPRD in hematopoiesis was investigated by analyzing the gene expression in human hematopoietic cells and by studying morphological changes in hematopoietic tissues in the *Ptprd* knockout mouse model.

B. Study the role of PTPRD as a tumor suppressor in melanoma

PTPRD is frequently mutated (in 12% of the cases) in melanoma, but it remains unclear whether the mutations identified are true loss-of-function mutations or rather passenger mutations induced by UV exposure. To answer this question, I performed a comprehensive mutation and expression analysis. To identify unknown substrates of PTPRD and to define the phosphatase activity of the PTPRD mutants identified in melanoma, I performed transfections and protein analysis in 293T cells.

3. Identify other signaling pathways controlled by PTPN2 in T-ALL

PTPN2 is already validated as a tumor suppressor gene in T-ALL, where it negatively regulates the JAK/STAT pathway. It was my aim to identify other signaling pathways affected by loss of PTPN2 in T-ALL using phospho-proteomics. Of the identified proteins, I focused on PTK2B, a tyrosine kinase that is known to be functioning downstream of the IL-7 receptor pathway. Since the IL-7 receptor pathway is involved in T-cell proliferation, I investigated the link between PTK2B and the IL7-R pathway and in which cellular response PTK2B is involved.

CHAPTER III – MATERIALS AND METHODS

Cell lines and patient samples

Expression analysis and CNV were analyzed in 57 human melanoma samples (UZ Leuven – KULeuven), 12 mouse melanoma samples (VIB – KULeuven), 5 human melanoma cell lines, 13 B-ALL samples (UMC St Radboud) and 18 ALL cell lines. All human samples were obtained according to the guidelines of the ethic committee (S55073). Functional studies were performed in 293T cells, the IL-3 dependent mouse pro-B cell line Ba/F3 and the IL-2/IL-7 dependent mouse T cell line MOHITO. The 293T cells were cultured in RPMI-1640 medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Life Technologies), the Ba/F3 cells in RPMI-1640 medium supplemented with 10% FBS and 1 ng/ml IL-3 (Peprotech Inc.), and the MOHITO cells in RPMI-1640 medium supplemented with 20% FBS and 5 ng/ml IL-2 and 10 ng/ml IL-7 (Peprotech Inc.). Ba/F3 cells and MOHITO cells transformed by oncogenic kinases did not require cytokines for their growth.

Histopathological analysis

The spleen tissues of the mice were isolated and overnight incubated with formaline (Sigma-Aldrich). After a washing step with PBS (Life Technologies), the tissue sections were stained with hematoxylin and eosin (H&E) according to standard procedures by the lab of prof. T. Tousseyn. Histological images were obtained using the Leica DM2500 microscope (Leica microsystems).

qPCR and qRT-PCR

RNA and DNA were extracted from patient samples, tissues and cell lines using the Maxwell 16 Instrument System (Promega). Tissues were first homogenized with the TissueLyser TL (Qiagen). cDNA was synthesized from the RNA samples with the GoScript Reverse Transcriptase (Promega). GoTaq qPCR Master Mix (Promega) was used for the performance of quantitative (real-time) PCR with the LightCycler 480 Real-Time PCR system (Roche Diagnostics) or the ViiA (Applied Biosystems). Data were analyzed with the qBase+ software (Biogazelle).

Analysis of cancer genomics datasets

CNV analysis of publically available array comparative genomic hybridization (CGH) datasets was performed with the Agilent genomic workbench software and with the OmicSoft Array Studio software. Mutation analysis of the publically available melanoma datasets was performed with the cBioPortal online web tool from the TCGA group. Gene expression profiles were studied with the cBioPortal, the Oncomine and the GeneVestigator online available web tools.

Constructs

A myc-tagged truncated version of PDGFRα (tPDGFRα) was cloned in the MSCV-puro vector (Clontech). The coding sequences of mouse *Pten* and *Cdkn2a* (p16) were amplified by PCR and cloned into the MSCV-puro vector containing already tPDGFRα. The CmiR3 vector was generated by cloning the mir30 backbone in the MSCV-GFP vector. The PTPRD constructs were purchased from Addgene.

shRNA and siRNA

The shRNA-Pten-4 was purchased from Open Biosystems. All the other shRNAs targeting *Pten* and *p16* were designed using the online available RNAi Central (http://katahdin.cshl.org/homepage/siRNA/RNAi.cgi?type=shRNA) and the oligonucleotide sequences were purchased from Integrated DNA Technologies (IDT). All shRNAs were cloned into the CmiR3 vector. The siRNAs targeting *Pten* and *p16* named with a letter were pre-designed RNAi molecules purchased from IDT. The siRNAs named with a number were custum-synthesized RNAi molecules designed with the RNAi design tool from IDT. The siRNA Ptk2b-A and the siRNA Jak1 were purchased from Ambion and the siRNA Ptk2-B from Invitrogen. As negative control, scrambled siRNAs from the same companies were used. The sequences of the shRNAs and siRNAs are listed in Table 1 and 2.

Table 1. Sequences shRNAs

shRNA	Top sequence
Pten-2	AACAATGAACCTGATCATTATATAGTGAAGCCACAGATGTATATAATGATCAGGTTCATTGTC
Pten-3	CTCTGTGAAGATCTTGACCAATTAGTGAAGCCACAGATGTAATTGGTCAAGATCTTCACAGAA
Pten-4	CCGACTTAGACTTGACCTATATTAGTGAAGCCACAGATGTAATATAGGTCAAGTCTAAGTCGA
p16-1	CGCAGCTCTTCTGCTCAACTACTAGTGAAGCCACAGATGTAGTAGTTGAGCAGAAGAGCTGCT
p16-2	ATTGGTCACTGTGAGGATTCAGTAGTGAAGCCACAGATGTACTGAATCCTCACAGTGACCAAG
p16-3	AGCTCTGGCTTTCGTGAACATGTAGTGAAGCCACAGATGTACATGTTCACGAAAGCCAGAGCG

Table 2. Sequences siRNAs

siRNA	Top sequence	Bottom sequence
PDGFRα	GGAUUCUACUUUCUACAAUAAGATC	GAUCUUAUUGUAGAAAGUAGAAUCCAC
Pten-A	UGAUAUCAAAGUAGAGUUCUUCCAC	GUGGAAGAACUCUACUUUGAUAUCACC
Pten-1	GGUGUAUACAGGAACAAUAUUGATG	CAUCAAUAUUGUUCCUGUAUACACCUU
Pten-2	GAGGCUAGCAGUUCAACUUCUGUGA	UCACAGAAGUUGAACUGCUAGCCUCUG
Pten-3	AGCUAAAGGUGAAGAUAUAUUCCTC	GAGGAAUAUAUCUUCACCUUUAGCUGG
Pten-4	GGGACGGACUGGUGUAAUGAUUUGT	ACAAAUCAUUACACCAGUCCGUCCCUU
Pten-5	CCCACCACAGCUAGAACUUAUCAAA	UUUGAUAAGUUCUAGCUGUGGUGGGUU
Pten-6	GGGUAAAUACGUUCUUCAUACCAGG	CCUGGUAUGAAGAACGUAUUUACCCAA
Pten-7	CAGCCAUCAUCAAAGAGAUCGUUAG	CUAACGAUCUCUUUGAUGAUGGCUGUC

Pten-8	GCCUGUGUGUGGUGAUAUCAAAGTA	UACUUUGAUAUCACCACACACAGGCAA
Pten-9	GGGAUUUCCUGCAGAAAGACUUGAA	UUCAAGUCUUUCUGCAGGAAAUCCCAU
p16-A	UCAAGACAUCGUGCGAUAUUUGCGT	ACGCAAAUAUCGCACGAUGUCUUGAUG
p16-B	GGCAACGUUCACGUAGCAGCUCUTC	GAAGAGCUGCUACGUGAACGUUGCCCA
p16-C	CAACUACGGUGCAGAUUCGAACUGC	GCAGUUCGAAUCUGCACCGUAGUUGAG
p16-D	ACGGGCAUAGCUUCAGCUCAAGCAC	GUGCUUGAGCUGAAGCUAUGCCCGUCG
p16-E	CUACCUUCUCCCGCCCGGUGCACGA	UCGUGCACCGGGCGGGAGAAGGUAGUG
p16-1	GCUCAACUACGGUGCAGAUUCGAAC	GUUCGAAUCUGCACCGUAGUUGAGCAG
p16-2	AGGCUAGAGAGGAUCUUGAGAAGAG	CUCUUCUCAAGAUCCUCUCUAGCCUCA
p16-3	GCAGGUUCUUGGUCACUGUGAGGAT	AUCCUCACAGUGACCAAGAACCUGCGA
p16-4	CUCUGGCUUUCGUGAACAUGUUGTT	AACAACAUGUUCACGAAAGCCAGAGCG
p16-5	UCCUGGACCAGGUGAUGAUGAUGGG	CCCAUCAUCAUCACCUGGUCCAGGAUU
Jak1	CCAGUGGCGGCAGAAACCAAAUGUU	AACAUUUGGUUUCUGCCGCCACUGG
Ptk2b A	GGGAUAUUGCUGUCCGGAATT	UUCCGGACAGCAAUAUCCCTG
Ptk2b B	AGCUGGGACACUACCUGGAACGAAA	UUUCGUUCCAGGUAGUGUCCCAGCU

Transfection of 293T cells

Transfection of 293T cells was performed to study protein signaling and to produce retroviral vectors. Therefore the cells were seeded out at a density of 0,6 x 10^6 cells in a 6-well plate and the transfection mix was added when the cell reached 50-70% confluency. The transfection mix contained 400 µl serum-free medium, 2 µg DNA and 4 µl Turbofect transfection reagent (Fermentas). After 20 minutes of incubation, the transfection mix was added drop wise to the medium of the 293T cells. The cells or viral vectors were harvested 48 hours after transfection.

Retroviral transduction

For a stable expression of our transgenes in Ba/F3 and MOHITO cells, we first cloned our genes of interest into Murine Stem Cell Virus (MSCV) retroviral expression vectors containing a GFP or puromycin selection marker. The 293T cells secrete the viral vectors in their medium after co-transfection of the retroviral vectors and an ecotropic packaging vector. The viral supernatant is harvested and filtered with acrodisc 0,45 µm filters (VWR) 48 hours after transfection. Transduction of the mouse cells with these vectors leads to a stable integration of the vector in the genome. For the transduction of Ba/F3 cells, 1 ml of viral supernatant was added to 1 x 10^6 cells in 1 ml of growth medium supplemented with 8 µg/ml Polybrene (Sigma-Aldrich) and IL-3 (Peprotech Inc.). After 24 hours, the cells were washed and the percentage GFP+ cells was measured by flow cytometry or cells containing the puromycin resistance gene were selected by adding 1 µg/ml puromycin to the medium. For the transduction of MOHITO cells, non-tissue treated 6-well plates were coated with RetroNectin (TAKARA Bio Inc.) and the retroviral supernatant was bound to the plate surface by

centrifugation (1000g, 30°C, 2 hours). After virus binding and removal of the supernatant, 1 x 10^6 MOHITO cells in 2 ml growth medium supplemented with 5 ng/ml IL-2 and 10 ng/ml IL-7 (Peprotech Inc.) were added to the plate followed by a spinoculation step (2000g, 30°C, 1 hour) to increase the contact between virus and cell. After recovery, the cells were removed from the RetroNectin plates and the GFP percentage was determined or puromycin selection was performed as described above.

Electroporation and siRNA-mediated knockdown

Cells were resuspended in 400 µl of serum-free growth medium, which contained 300-400 nM siRNA, and transferred to 4-mm cuvettes (Bio-Rad). The amount of cells, the concentration of the siRNA and the electroporation parameters differ for each specific cell line used (Table 3). The electroporated cells were transferred to 12-well plates containing 2ml pre-warmed growth medium. Knockdown efficiency is analyzed through western blotting or qRT-PCR. Growth reduction was analyzed by measuring the amount of viable cells 1 hour and 48 hours after electroporation using the GUAVA flow cytometer (Millipore). The siRNAs targeting the gene of interest and their negative controls were purchased from IDT and Invitrogen.

Table 3. Electroporation parameters for cell lines

Cell line	Amount of cells	Amount of siRNA	Electroporation parameters
MOHITO	1,5 x 10^6	300 nM	Exponential decay, 350 V, 500 µF
Ba/F3	1 x 10^6	400 nM	Exponential decay, 300 V, 950 µF

Western blotting

Cells were lysed in cold lysis buffer (Cell Signaling Technology – Bioké) supplemented with protease inhibitors (cOmplete Protease Inhibitor Cocktail tablets). Protein concentrations were determined using the Bio-Rad protein assay, which measured the sample absorbance at 595nm. Next the proteins were denaturated and reduced on 70°C for 10 minutes in NuPAGE LDS sample buffer and NuPAGE Reducing agent (Life Technologies). The proteins were separated on NuPAGE NOVEX Bis-Tris 4-12% gels (Invitrogen) and transferred to PVDF membranes (GE Healthcare). Subsequent western blot analysis was performed using primary antibodies (Table 4) and the secondary antibodies sheep anti-mouse IgG-HRP, donkey anti-rabbit IgG-HRP (GE Healthcare) and donkey anti-goat IgG-HRP (Santa Cruz Biotechnology/SCBT). For the signal detection, the membranes were treated for weak signals with the Amersham ECL Prime Western Blotting detection reagent (GE Healthcare) and for strong signals with the Western Lightning Plus ECL (Perkin Elmer) and the signals were visualized on the Image Quant LAS 4000 mini imager (Fujifilm).

Table 4. Primary antibodies

Antibody	Ref. Number	Company
b-actin	A1978	Sigma-Aldrich
Phospho-CBL	#8869	Cell Signaling Technology
ERK1/2	Sc-93	Santa Cruz Biotechnologies
Phospho-ERK1/2 (Thr202/Tyr204)	#4377	Cell Signaling Technology
JAK1	05-1154	Millipore
Phospho-JAK1 (Tyr1022/1023)	sc-16773R	Santa Cruz Biotechnologies
LCK	sc-13	Santa Cruz Biotechnologies
Phospho-LCK (Tyr394)	SAB4300118	Sigma-Aldrich
Phospho-LCK (Tyr505)	#2751	Cell Signaling Technology
MEK1/2	#9126	Cell Signaling Technology
Phospho-MEK1/2 (Ser217/221)	#9154	Cell Signaling Technology
PDGFRα	sc-338	Santa Cruz Biotechnologies
Phospho-PDGFRα	sc-12911-R	Santa Cruz Biotechnologies
PTEN	sc-7974	Santa Cruz Biotechnologies
PTPRD	sc-10867	Santa Cruz Biotechnologies
PTK2B	#3480	Cell Signaling Technology
Phospho-PYK2 (Tyr402)	#3291	Cell Signaling Technology
Phospho-SRC family	#2101	Cell Signaling Technology
STAT1	#9172	Cell Signaling Technology
Phospho-STAT1 (Tyr701)	#9167	Cell Signaling Technology
STAT3	#4904	Cell Signaling Technology
Phospho-STAT3 (Tyr705)	#9138	Cell Signaling Technology
STAT5	#9358	Cell Signaling Technology
Phospho-STAT5 (Tyr694)	#9359	Cell Signaling Technology

Cytokine stimulation experiments

Cytokine dependent cell lines were washed thrice with PBS (Life Technologies) to remove the cytokines and starved for 3 hours in their growth medium without cytokines. For each condition 4 x 10^6 cells were stimulated with 5 ng/µl IL-2 and 10 ng/µl IL-7 during 10-20 minutes. In some conditions 100 nM final concentration of the SRC inhibitors, AZD0530 (Selleckchem) and dasatinib (Selleckchem), were added. As a negative control for the used inhibitors, the addition of dimethyl sulfoxide (DMSO) was used.

Inhibitor assay

To obtain dose-response curves, MOHITO cells were seeded in five-fold in 96-well plates at a concentration of 3 x 10^5 cells in a total volume of 100 µl and JAK inhibitors, ruxolitinib (Axon Medchem) and tofacitinib (Selleckchem), or SRC inhibitors, AZD0530 (Selleckchem) and dasatinib (Selleckchem), were added with increasing concentrations. As a negative control, the addition of DMSO was used. The amount of viable cells was measured after seeding and after an incubation time of 24 and 48 hours by measuring the luminescence on a Victor X4 plate reader (Perkin-Elmer) after adding ATPlite (Perkin-Elmer).

Migration assay

For the migration assay, ThinCerts with a pore size of 8 µm (Greiner Bio-One) were placed in wells of a 24-well plate. To the ThinCerts, 2 x 10^5 MOHITO cells resuspended in 100 µl growth medium, containing 5% FBS, 5 ng/ml IL-2 and 10 ng/ml IL-7, was added. To the wells, 600 µl of 20% FBS growth medium supplemented with 5 ng/ml IL-2 and 10 ng/ml IL-7 was added. A final concentration of 100 nM of AZD0530 or dasatinib was added to the medium in the insert and the well. To explore how many cells after an incubation time of 6 hours migrated through the membrane of ThinCert to the well, the amount of viable cells in the well and in the ThinCert was measured with the GUAVA flow cytometry. The percentage cell invasion was calculated according to the calculation: cell invasion (in %) = N lower/(N lower + N upper) x 100

Phospho–proteomics

To identify the signaling pathways controlled by PTPN2, we performed the Stable Isotope Labeling by Amino Acids in Cell culture (SILAC) phospho-proteomics method. Untransduced MOHITO cells were grown in light RPMI SILAC labeled media containing L-Lysine and L-Arginine amino acids. MOHITO cells with knockdown of PTPN2 were grown in heavy RPMI SILAC labeled media containing [U-$^{13}C_6$]-L-Lysine and Arg10 L-Arginine heavy labeled amino acids. After 6 doubling times, 100 x 10^6 cells of each culture were lysed with a freshly made cell lysis buffer, containing 50 mM Tris (pH 7,5), 150 mM NaCl, 1% NP-40, 0,25% Na, deoxycholate, protease inhibitor cocktail, 1mM Na_3VO_4, 5 mM β-glycerophosphate and 5 mM NaF. Next, the cell lysates of both cultures were mixed in a 1:1 ratio. To isolate the tyrosine-phosphorylated proteins from the cell lysate immunoprecipitation was performed with Dynabeads Protein G (Life Technologies) and the 4G10 (Upstate) and PY100 (Cell Signaling Technologies) anti-phospho-tyrosine antibodies. After immunoprecipitation the proteins in the cell lysates were separated by electrophoresis on a NuPAGE NOVEX Bis-Tris 4-12% gel. After visualization of the separated proteins with coomassie staining, the gel was cut in smaller pieces for in-gel digestion, which was performed as described previously[132]. The fragmented peptides were next analyzed on the LTQ Orbitrap mass spectrometry by the group of prof. Dr. Blagoy in Denmark.

CHAPTER IV - RESULTS

1 Development of a siRNA and shRNA screening system based on a kinase fusion protein

The obtained results described in this part have been published as:
Pieraets S, Cox L, Gielen O and Cools J. Development of a siRNA and shRNA screening system based on a kinase fusion protein. *RNA* (2012), 18:1296-1306.

ABSTRACT

RNA interference (RNAi) is one of the processes in the cell that regulates mRNA expression levels. RNAi can be exploited to experimentally knockdown the expression of one or more genes in cell lines or even in cells *in vivo* and became also an interesting tool to develop new therapeutic approaches. One of the major challenges of using RNAi is selecting effective shRNAs or siRNAs that sufficiently downregulate the expression of the target gene. Here, we describe a system to select functional shRNAs or siRNAs that makes use of the leukemia cell line Ba/F3 that is dependent on the expression of a mutant form of the PDGFRα kinase for its proliferation and survival. The basis of this system is the generation of an expression construct where part of the open reading frame of the gene of interest is linked to the mutant PDGFRα. Thus, shRNAs or siRNAs that effectively target the gene of interest also result in a reduction of the expression of the mutant PDGFRα protein, which can be detected by a reduction of the proliferation of the cells. We demonstrate that this validation system can be used for the selection of effective siRNAs as well as shRNAs. Unlike other systems, the system described here is not dependent on obtaining high transduction efficiencies, and non-specific effects of the siRNAs or shRNAs can be detected by comparing the effects in the presence or absence of the growth factor interleukin-3.

INTRODUCTION

RNA interference (RNAi) is the process of gene silencing that occurs in a sequence specific manner at the level of the mRNA[133]. The basic process of RNAi can be divided into three steps. Initially, the ribonuclease III enzyme Dicer cleaves a long double stranded RNA, which is expressed or introduced into the cell, into small RNAs (20-30 nucleotides). Secondly, one strand of the small RNA, the guided strand, is loaded onto the RNA-induced silencing complex (RISC), while the other, the passenger strand, is degraded. Finally, the guided strand binds the target mRNA, which results in the degradation of the mRNA. Human cells express hundreds of non-coding RNAs that are processed in the cell to short RNAs that enter the RNAi pathway and are important for gene expression regulation[134-136]. Synthesized double stranded short interfering RNAs (siRNAs) introduced into the cell or short hairpin RNAs (shRNAs) or miRNA-based shRNAs (shRNAmirs) expressed in the cell use the cellular mechanism of RNAi to interfere with the expression of a specific gene and in this way create a cell that lacks sufficient expression levels of that gene[133-135,137].

In the lab RNAi is used as a research tool in the siRNA and shRNA/shRNAmir mode. Such siRNAs are ~21-25 nucleotides long and are introduced artificially by means of transfection or electroporation. The shRNAs and shRNAmirs are typically incorporated in expression vectors such as plasmids or retroviral/lentiviral vectors, through which the shRNAs or shRNAmirs are stably integrated in the host genome. Those synthesized small RNA molecules use the endogenous RNAi pathway in eukaryotic cells for the degradation of their target mRNA[133-135,137,138].

Despite the general use of RNAi in research, there are still a number of limitations and difficulties associated with this technology. First of all, delivery of siRNAs to cells *in vitro* and even more *in vivo* remains a bottleneck for the widespread use of RNAi. Second, despite the specificity due to the sequence of the siRNAs or shRNAs, off-target effects are present and may cause unwanted and difficult to control side effects. Third, the selection of siRNA or shRNA sequences that are effective in downregulating the expression of the target genes remain difficult[138-142]. Although design algorithms have improved in the last years, there are still difficulties in identifying effective and specific siRNAs and shRNAs. Therefore, it is still required to experimentally validate every siRNA or shRNA that is used, and for a specific gene, 10 or more sequences may need to be tested to identify one RNAi molecule that is causing sufficient knockdown[143-145].

To assist in the identification of functional siRNAs or shRNAs, a number of experimental validation systems have been developed. Those validation systems consist of a reporter assay where a target gene is fused to a fluorescent or enzymatic reporter. In these systems, expression of functional shRNAs or siRNAs results in a decrease of the reporter signal, and thus loss of fluorescent or enzymatic activity is a measure for the knockdown efficiency of the siRNAs or shRNAs[146-153].

In this work, we describe the development of a protein-kinase fusion system in Ba/F3 cells that is based on a proliferation read-out to test the knockdown efficiency of RNAi molecules.

RESULTS

Development of the Ba/F3 validation system

In order to evaluate the silencing capacity of selected shRNAs or siRNAs in a medium- to high-throughput system, we developed a cell-based system in which shRNA/siRNA molecules that efficiently knockdown the expression of a given gene would inhibit cell proliferation. In this way, the silencing effect can be read from the effect on cell proliferation, as is also done in cell-based drug screens (Fig. 13). For this, we used the interleukin-3 (IL-3) dependent pro-B-cell line (Ba/F3). These Ba/F3 cells are dependent on IL-3 for their proliferation and survival, but can also become dependent on oncogenic tyrosine kinases such as activated ABL, FLT3, PDGFRα, and many other kinases[154,155]. For our system, we used a truncated form of PDGFRα (named “tPDGFRα”), known to be constitutively activated and to stimulate the proliferation and survival of Ba/F3 cells in the absence of IL-3[30-32,155].

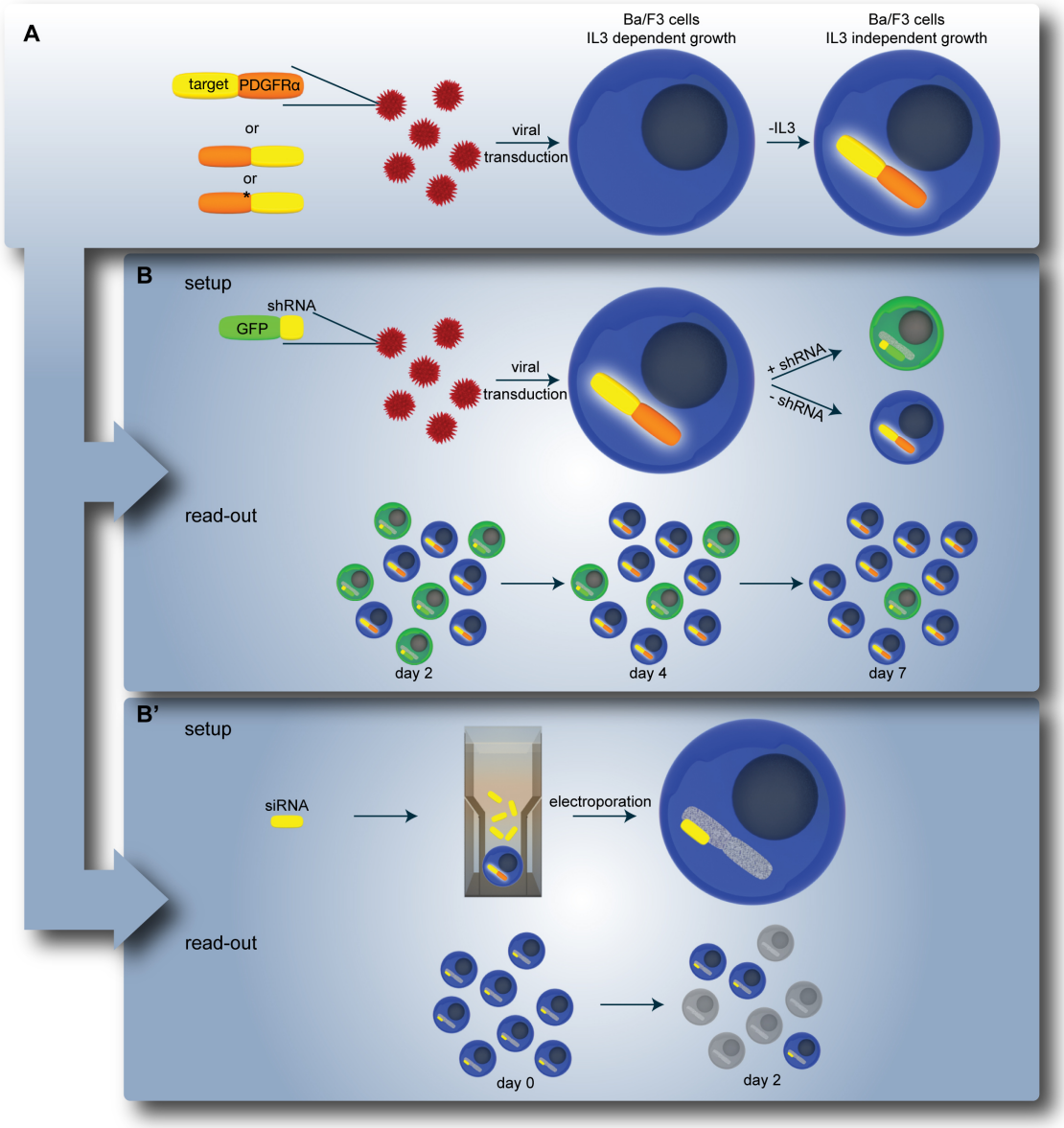


Figure 13. Schematic overview of the use of the validation system for selecting functional shRNAs or siRNAs (A) Ba/F3 cells expressing tPDGFRα or a fusion of tPDGFRα with a gene of interest can grow in the absence of the growth factor IL-3. These Ba/F3 cells are completely dependent on the expression of the mRNA containing the *tPDGFRα* open reading frame. The gene of interest is placed on the same mRNA, either upstream or downstream of the *tPDGFRα*. The asterix indicates the presence of a stop codon. (B) Transduction of the Ba/F3 cells with shRNAs (co-expressing GFP) that effectively downregulate the gene of interest and thus prevent expression of the tPDGFRα, will result in a decrease of the number of GFP positive cells in the culture. (B') Electroporation of Ba/F3 cells with siRNAs that effectively downregulate the gene of interest and thus prevent expression of the tPDGFRα, will inhibit the proliferation of the cells.

Based on our previous experiences with the Ba/F3 cell system, we reasoned that this cell system would be a good screening system to select efficient siRNA or shRNA sequences. Ba/F3 cells transformed by tPDGFRα have a short doubling time and are absolutely dependent on the activated kinase for their proliferation and survival. A siRNA targeting *tPDGFRα* can be used as a positive control in the screens, and IL-3 can be added to the cells to make them independent of tPDGFRα in order to distinguish between specific and toxic off-target effects of the siRNAs or shRNAs. Finally, the *tPDGFRα* can be fused to additional sequences without affecting its function[32]. In this way, the gene of interest (the gene that one wants to target) can be put on the same transcript as *tPDGFRα* (Fig. 13, 14A).

Three different variants of the vector were generated: variant 1 with the target gene upstream of *tPDGFRα*, generating a fusion protein between the coding sequence of the target gene and *tPDGFRα*; variant 2 with the target gene downstream of *tPDGFRα* with a fusion between *tPDGFRα* and the coding sequence of the target gene; and variant 3 with the target gene downstream of *tPDGFRα* on the same RNA, but without generating a fusion protein (Fig. 14A). Thus, the first two variants result in the expression of a fusion protein of tPDGFRα with the protein of interest (or a part of the protein of interest), while in the third version the target gene is present as the 3' untranslated region (UTR) of *tPDGFRα* (Fig. 13, 14A). To keep the ATG context the same for each construct a MYC-tag was cloned in front of each transcript. In that way we reduce variations in transcription among the different construct to a minimum.

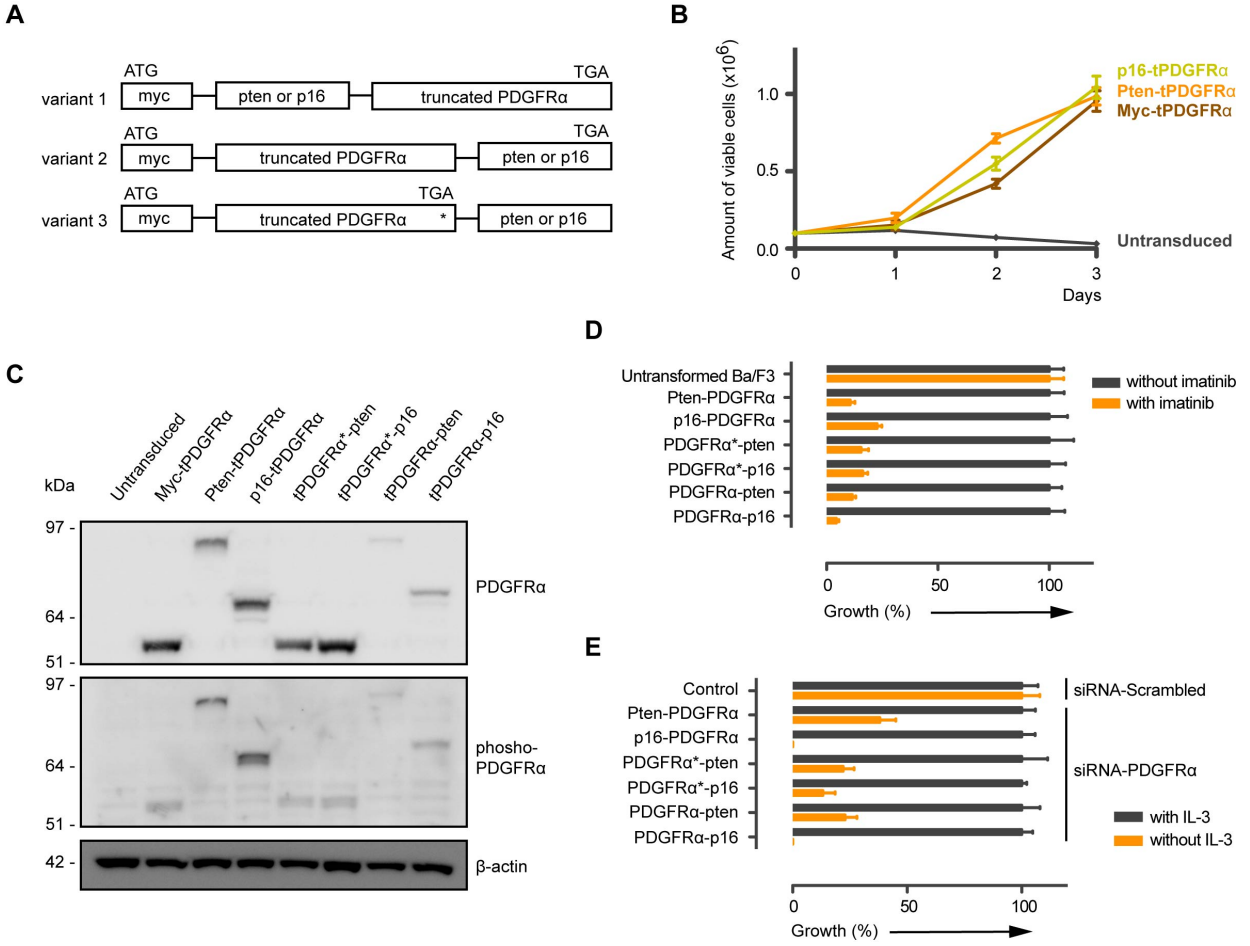


Figure 14. Expression of all constructs containing the *tPDGFRα* in Ba/F3 cells results in IL-3 independent growth (A) Schematic representation of the different constructs: myc-Pten-tPDGFRα, myc-p16-tPDGFRα, myc-tPDGFRα-Pten and myc-tPDGFRα-p16, myc-tPDGFRα*-Pten and myc-tPDGFRα*-p16. The target genes *Pten* and *p16* were cloned upstream of the *tPDGFRα*, downstream and downstream with a stop codon (*) in between. (B) Ba/F3 cells transduced with the *tPDGFRα* constructs grow in the absence of IL-3. Untransduced Ba/F3 cell do not survive without IL-3. Mean growth ± SEM was recorded in triplicate for 3 days. (C) All transformed Ba/F3 cells showed expression (upper panel) and phosphorylation (central panel) of the tPDGFRα. Expression of β-actin (lowest panel) is used as control for the loading. (D) Compared to the untransformed cells, growth of the transformed Ba/F3 cells was reduced when treated with 100 nM of the PDGFRα kinase inhibitor imatinib. Mean growth ± SEM was recorded in triplicate on day 0 and 2. (E) The transformed Ba/F3 cells became again dependent on IL-3 for their cell growth after electroporation with an effective siRNA targeting PDGFRα. As control, all cell lines were electroporated with a scrambled siRNA. Mean growth ± SEM was recorded in triplicate on day 0 and 2. Data of the scrambled siRNA are set to 100% and data of the tested siRNAs are relative to the scrambled siRNA.

Selection of shRNAs targeting *p16* and *Pten* using the Ba/F3 system

In order to test the Ba/F3 system, we designed shRNAs targeting mouse *Pten* and mouse *Cdkn2a* (*p16*), and tested if the Ba/F3 system could be used to select the most potent shRNA. We generated *tPDGFRα* constructs containing the coding sequences of *Cdkn2a* (*p16*) and *Pten* (Fig. 14A). These constructs were retroviral transduced in Ba/F3 cells. As such, six different Ba/F3 cell lines were generated to test the system. All six newly generated Ba/F3 cell lines could grow in the absence of IL-3 at the same proliferation rate as the control cells expressing only the truncated form of PDGFRα (Fig. 14B). Western blot analysis revealed auto-phosphorylation of all tPDGFRα fusion proteins in these different cell lines, indicating that all cell lines expressed activated PDGFRα as expected (Fig. 14C). In addition, the growth of all cell lines was inhibited by the PDGFRα kinase inhibitor imatinib, further confirming that the cell lines were all strictly dependent on the expression of the activated PDGFRα (Fig. 14D). In another experiment we showed that after electroporation with an effective siRNA against *tPDGFRα* those cell lines were again dependent on IL-3 for their cell growth (Fig. 14E).

To test the potency of the different retroviral shRNA vectors, we transduced the variant Ba/F3 cells with these retroviral vectors and followed the effect on the proliferation of the transduced cells. Transduction efficiencies between 40 and 70% were obtained. Since the shRNA vectors also express green fluorescent protein (GFP), we could identify shRNA-expressing cells using flow cytometry, which allowed us to trace the fate of the transduced cells over time. If the percentage GFP positive cells decreased over time, this was indicative of effective knockdown of the target RNA, since the target RNA is on the same RNA as *tPDGFRα*, which is required for the proliferation of the cells (Fig. 13).

In a first set of experiments we used the Ba/F3 cell lines with the open reading frames of *p16* or *Pten* positioned upstream of *tPDGFRα* and could demonstrate a clear distinction between effective and non-effective shRNAs. In the cells expressing p16-tPDGFRα the strongest decrease of GFP positive cells was obtained with shRNA-p16-1. ShRNA-p16-3 was less efficient and no effect was observed for shRNA-p16-2 at the endpoint of the experiment, 11 days after transduction. No significant change in the GFP positive population was observed in the control cells that expressed tPDGFRα or the cells containing the Pten-tPDGFRα fusion (Fig. 15A).

When targeting the cells expressing Pten-tPDGFRα, all three shRNAs targeting *Pten* were found to be effective, with the strongest effect observed for shRNA-Pten-4. To rule out that the effects observed on the proliferation of the Ba/F3 cells was due to more general toxic effects, we also tested the Pten shRNAs in parental Ba/F3 cells and Ba/F3 cells expressing p16-tPDGFRα. The different Pten shRNAs had no effect on the control cells or on the cells containing the p16-tPDGFRα fusion confirming that the observed effects were specific (Fig. 15A).

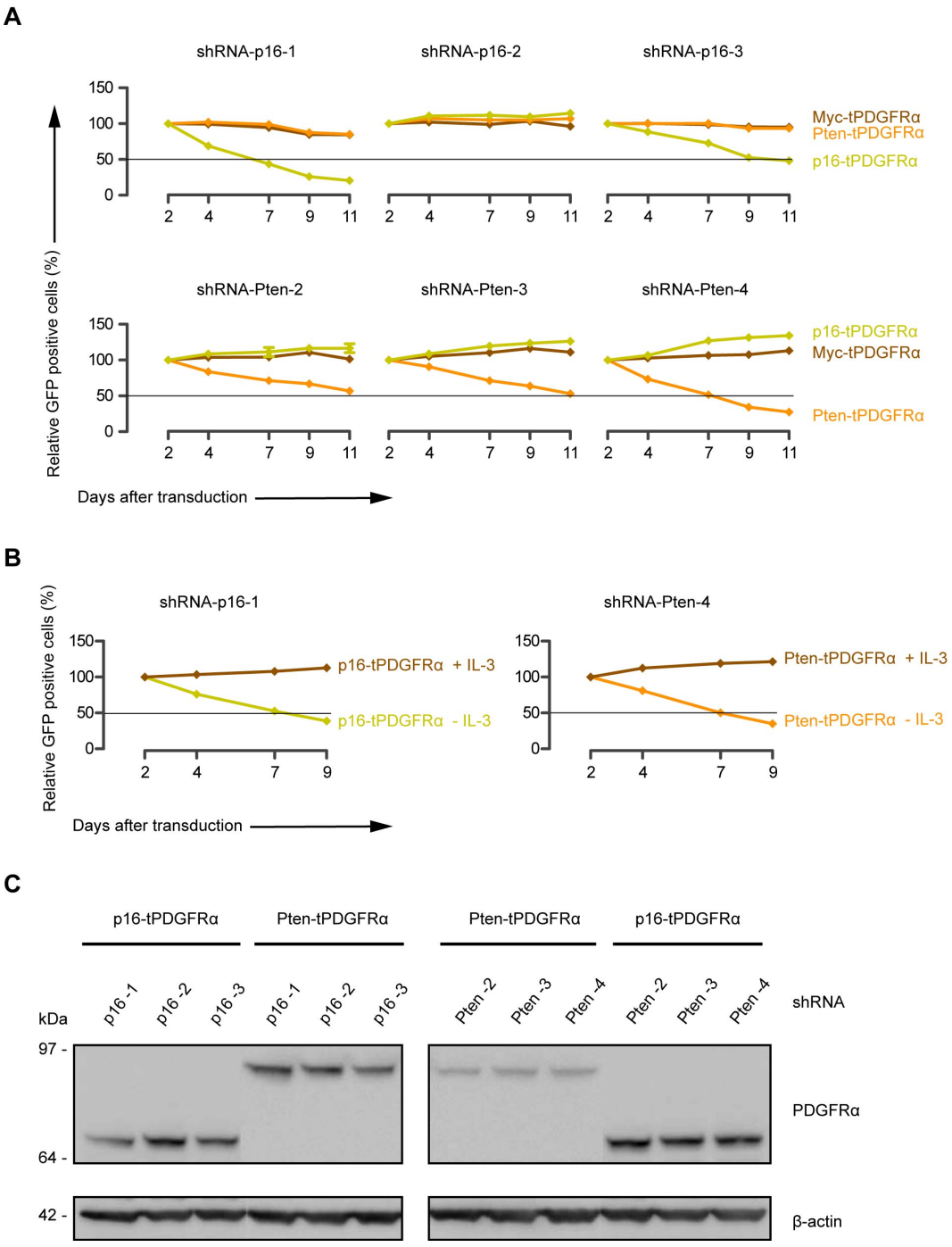


Figure 15. Screening of shRNAs using Pten-tPDGFRα and p16-tPDGFRα transformed Ba/F3 cells (A) Effective shRNAs lead to a decrease in the relative amount of GFP positive cells in contrast to non-effective shRNAs. Mean relative amount of GFP positive cells ± SEM was recorded in triplicate on day 2, 4, 7, 9 and 11. All values on day 2 are set to 100% and all other values are relative to day 2. (B) The proliferation of Ba/F3 cells expressing the p16-tPDGFRα or Pten-tPDGFRα fusion proteins were inhibited after expression of shRNA-p16-1 or shRNA-Pten-4, and this inhibition could be rescued by the addition of IL-3. Mean relative amount of GFP positive cells ± SEM was recorded in triplicate on day 2, 4, 7, 9 and 11. All values on day 2 are set to 100% and all other values are relative to day 2. (C) Downregulation of Pten-tPDGFRα and p16-tPDGFRα protein expression in transformed Ba/F3 cells transduced with shRNAs against *p16* and *Pten*. Cell lysates were obtained 4 days after transduction.

Toxic off-target effects of a shRNA can be identified by simply adding IL-3 to the transformed cells, which makes the cells independent from the tPDGFRα protein by stimulating their proliferation and survival through the IL-3 receptor. Specific shRNAs that target *p16* or *Pten* in the *tPDGFRα* transcripts should result in inhibition of cell proliferation, and this negative effect on proliferation should be rescued by the addition of

IL-3. Indeed, we showed that the cell lines, expressing the p16-tPDGFRα or Pten-tPDGFRα fusion proteins, could proliferate in the presence of IL-3 after transduction with the effective shRNA-p16-1 or shRNA-Pten-4, but not in the absence of IL-3 (Fig. 15B). These data confirm that the effects we observed of the shRNAs in the absence of IL-3 are not due to a nonspecific effect of the shRNAs.

The data of the proliferation assay were confirmed by protein expression analysis in transformed Ba/F3 cells containing p16-tPDGFRα transduced with the shRNAs targeting *p16*, and similarly in cells containing Pten-tPDGFRα transduced with shRNAs targeting *Pten*. As expected, the effective shRNA-p16-1 and -3 resulted in a significant decrease of the protein expression of p16-tPDGFRα, while there was no downregulation of p16-tPDGFRα protein levels by shRNA-p16-2 (Fig. 15C). Furthermore, the strongest downregulation of the Pten-tPDGFRα fusion gene was observed for shRNA-Pten-4. To a lesser extend shRNA-Pten-2 and -3 also resulted in decreased protein expression of Pten-tPDGFRα. There was no knockdown in the control experiments where Ba/F3 cells with p16-tPDGFRα were transduced with Pten shRNAs or where Ba/F3 cells with Pten-tPDGFRα were transduced with p16 shRNAs, indicating the specificity of the system (Fig. 15C).

For the experiments described above, we only used the constructs in which the gene of interest (*p16* or *Pten*) was cloned upstream of *tPDGFRα*, generating p16-tPDGFRα or Pten-tPDGFRα fusion proteins. Next, we determined if the system was also functioning in cases where the gene of interest was cloned downstream of *tPDGFRα*, either as fusion protein or downstream from the stop codon of tPDGFRα (thus in the 3' UTR of tPDGFRα). Our results show that the position of the target gene indeed matters. Although shRNA-p16-1 could potently decrease the proliferation of Ba/F3 cells expressing p16-tPDGFRα, much less effect was observed with the shRNA in cells expressing tPDGFRα-p16 or tPDGFRα*-p16. Similarly, shRNA-Pten-4 resulted in substantially lower decrease in the amount of GFP positive cells in experiments with Pten-tPDGFRα compared to tPDGFRα-Pten or tPDGFRα*-Pten (Fig. 16A). The same results were observed on protein level when the genes of interest were placed in front of *tPDGFRα*. After transduction with shRNA-p16-1 reduction in p16-tPDGFRα expression level was more pronounced compared to tPDGFRα-p16 or tPDGFRα*-p16. No knockdown of the control constructs containing *Pten* was observed with shRNAs targeting *p16*. Also, the protein level of Pten-tPDGFRα was more reduced after shRNA-Pten-4 transduction compared to tPDGFRα-Pten or tPDGFRα*-Pten. There was no knockdown of the control constructs containing *p16* with shRNAs targeting *Pten* (Fig. 16B). Similar results were obtained for the other shRNAs (data not shown). These data demonstrate that for the proliferation read-out, the constructs in which the gene of interest are positioned at the 5' end of the mRNA, upstream of *tPDGFRα*, are to be preferred. These results also confirm that we do not obtain similar knockdown effects for the three different variants of the system, and thus that knockdown efficiency is dependent on the position of the target sequence in the mRNA.

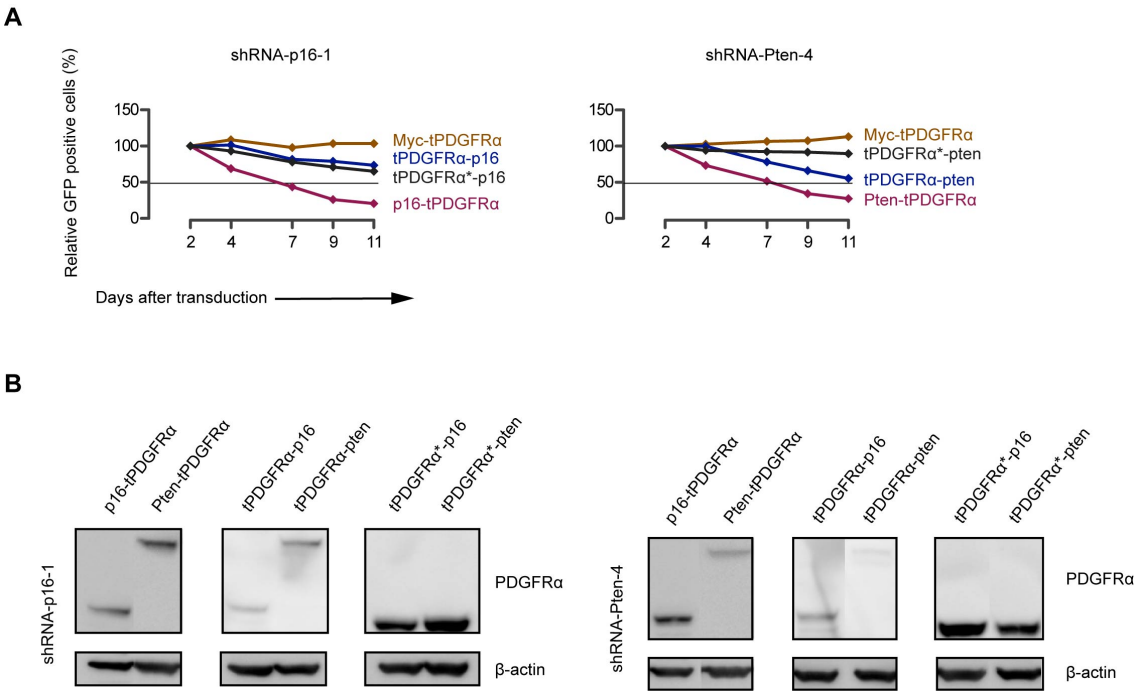


Figure 16. Screening of shRNAs directed against *Pten* and *p16* results in different outcomes according to the position of the gene of interest in the *tPDGFRα* constructs (A) Effective shRNAs result in a decrease of the relative amount of GFP positive cells when *Pten* and *p16* are placed upstream of the *tPDGFRα*. A less clear effect is observed when *Pten* and *p16* are downstream of the *tPDGFRα*. Mean relative amount of GFP positive cells ± SEM was recorded in triplicate on day 2, 4, 7, 9 and 11. All data on day 2 are set to 100%, and all other data are relative to day 2. (B) Downregulation of tPDGFRα and tPDGFRα fusion proteins in the transformed Ba/F3 cells transduced with shRNA-p16-1 or shRNA-Pten-4. Cell lysates were obtained 4 days after transduction.

Selection of siRNAs targeting *p16* and *Pten* using the Ba/F3 validation system

In addition to testing of shRNAs, we also applied our validation system for the selection of effective siRNAs by electroporation of the Ba/F3 cells (Fig. 13B'). We electroporated the Ba/F3 cells expressing p16-tPDGFRα or Pten-tPDGFRα with 10 siRNAs directed against *p16* and 10 siRNAs directed against *Pten*, respectively. Two days after electroporation, we measured the growth of the cells, which revealed major differences between the different siRNAs (Fig. 17A). Four of these siRNAs were tested in more detail (Fig. 17A, indicated in orange) and effects were confirmed in a second experiment (Fig. 17B). The growth reduction was the highest for siRNA-p16-3 and siRNA-Pten-9 respectively. SiRNA-p16-4, siRNA-p16-5, siRNA-Pten-1 and siRNA-Pten-2 were less effective in reducing the growth of the cells. The non-effective siRNA-p16-D and siRNA-Pten-A were not able to reduce the growth in the transformed Ba/F3 cells containing the p16-tPDGFRα or Pten-tPDGFRα respectively (Fig. 17B). As also observed for the shRNAs, the effect of the siRNAs on cell proliferation was less pronounced when *p16* and *Pten* were placed downstream of the *tPDGFRα* (data not shown).

As shown for the shRNA experiments, cell lines electroporated with siRNAs can be grown in the presence of IL-3 to distinguish between specific and toxic effects. The cell lines expressing p16-tPDGFRα or Pten-tPDGFRα survived after electroporation with an effective siRNA against *p16* or *Pten* when IL-3 was added (Fig. 17C). These data confirm that the siRNAs produce the inhibitory effect on proliferation of the cells specifically through the knockdown of the p16-tPDGFRα or Pten-tPDGFRα transcripts, and not due to non-specific knockdown of critical genes needed for proliferation or survival.

In general, the knockdown effect on protein expression (Fig. 17D) correlated well with the effect on cell proliferation (Fig. 17B). Indeed, clear downregulation of expression of the p16-tPDGFRα or Pten-tPDGFRα fusion proteins was observed when the cell lines were electroporated with the siRNAs that had a clear effect on the proliferation of the cells, while less or no knockdown was observed for siRNAs not having much effect on the proliferation (Fig. 17B and D). These data confirm the correlation between effects on protein expression and effects on cell proliferation in the Ba/F3 system.

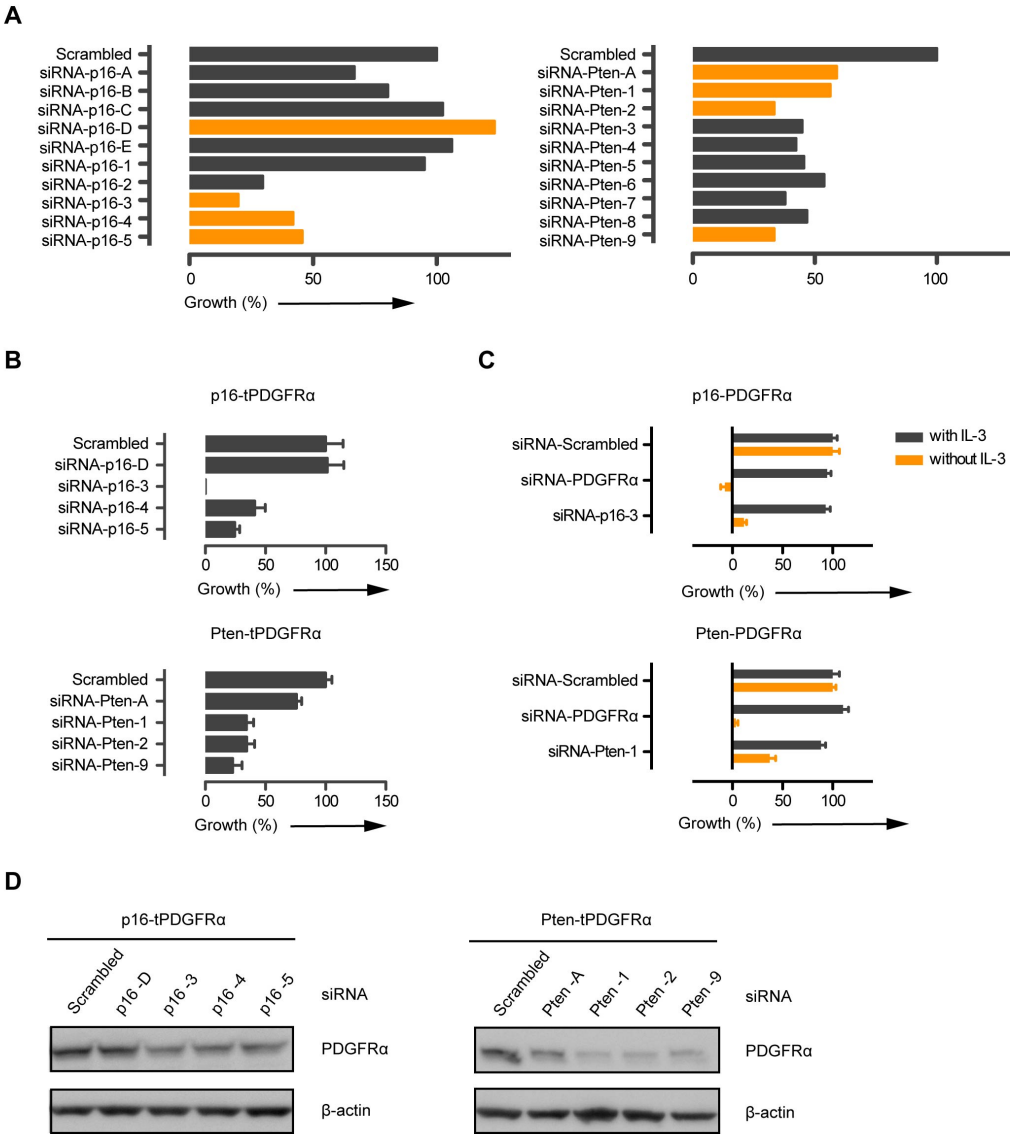


Figure 17. Screening and validation of siRNAs (A) Ten siRNAs directed against *Pten* and *p16* were screened in the p16-tPDGFRα and the Pten-tPDGFRα transformed Ba/F3 cells respectively. The siRNAs indicated in orange were confirmed in a second experiment (panel B). (B) Effective siRNAs result in reduced growth of p16-tPDGFRα and Pten-tPDGFRα transduced Ba/F3 cells. Mean growth ± SEM was recorded in five-fold on day 0 and 2. Data of the scrambled siRNA are set to 100%, data of the tested siRNAs are relative to the scrambled siRNA. (C) The proliferation of Ba/F3 cells expressing the fusion protein p16-tPDGFRα or Pten-tPDGFRα was inhibited after electroporation with the effective siRNA-p16-3 or siRNA-Pten-1, respectively, and this inhibition could be rescued by the addition of IL-3. As control, all cell lines were electroporated with a scrambled siRNA. Mean growth ± SEM was recorded in triplicate on day 0 and 2. Data of the scrambled siRNA are set to 100% and the data of the tested siRNAs are relative to the scrambled siRNA. (D) Effective siRNA gave a reduction in the protein expression of p16-tPDGFRα and Pten-tPDGFRα.

Correlation of the knockdown efficiency in the Ba/F3 cell system with knockdown of endogenous Pten or p16 expression in mouse cell lines

Previous experiments demonstrated the potential use of the Ba/F3 system to screen for functional siRNAs or shRNAs, but did not address the correlation between the results in the Ba/F3 cell system and the effects on the normal endogenous transcripts of *Pten* or *Cdkn2a*. To investigate if the results of the Ba/F3 system reflect the knockdown efficiencies obtained on endogenous transcripts, we next performed experiments with parental Ba/F3 cells and MOHITO cells[156]. We determined whether the effective shRNAs and siRNAs, validated in the system, were also able to downregulate endogenous *Pten* transcripts in Ba/F3 cells. Due to the absence of endogenous p16 expression in Ba/F3 cells we used endogenous *Cdkn2a* (*p16)* transcripts in MOHITO cells to validate the p16 RNAi molecules.

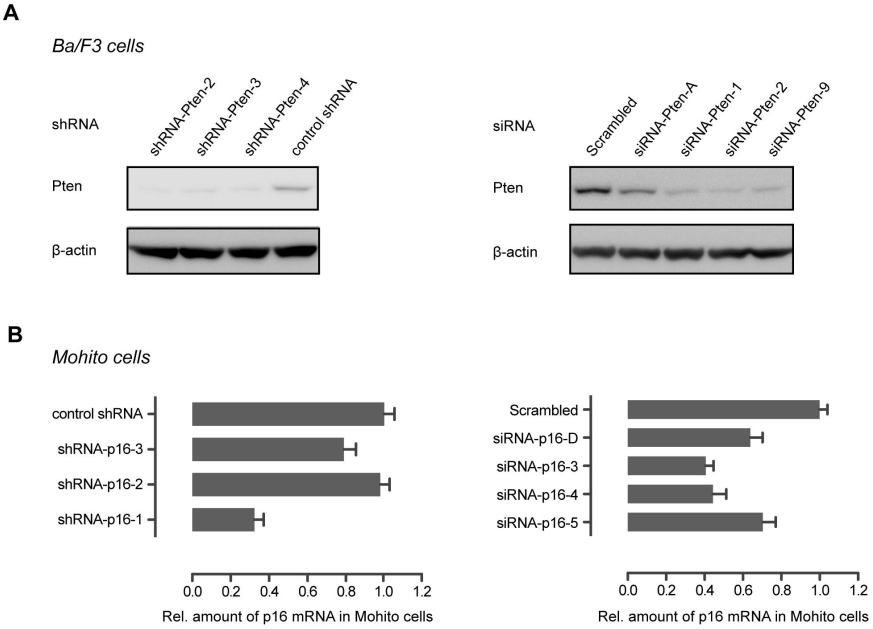


Figure 18. Downregulation of endogenous Pten and p16 in Ba/F3 cells and in MOHITO cells (A) Downregulation of endogenous Pten expression in Ba/F3 cells by shRNAs (left panel) and by siRNAs (right panel). The control shRNA mentioned in the left panel is shRNA-p16-1. (B) Reduction in endogenous *p16* transcripts in MOHITO cells by shRNAs (left panel) and by siRNAs (right panel). The control shRNA in the left panel is shRNA-Pten-4. Cell lysates and RNA of cells transduced with shRNAs were obtained 4 days after transduction and cell lysates and RNA of cells electroporated with siRNAs were obtained 2 days after electroporation.

Parental Ba/F3 cells were transduced with the shRNAs targeting *Pten*, and knockdown of *Pten* transcripts was measured by the detection of Pten protein expression. The three shRNAs produced strong knockdown of Pten (Fig. 18A, left panel), which correlated well with effects observed in the Ba/F3 validation system (Fig. 15), where also all three shRNAs showed strong effects with the shRNA-Pten-4 being the most potent. Decrease in the endogenous Pten expression was also observed when Ba/F3 cells were electroporated with the siRNAs targeting *Pten*. Compared to the scrambled siRNA, the decrease in expression was minimal when electroporated with the siRNA-Pten-A, and a strong knockdown was observed with siRNA-Pten-1, siRNA-Pten-2 and siRNA-Pten-9 (Fig. 18A, right panel). These data recapitulated well the data observed with the Pten-tPDGFRα expressing Ba/F3 cells (Fig. 17).

Similarly, also for the p16 shRNAs, a good correlation was observed between the knockdown of the *p16* transcript in MOHITO cells (Fig. 18B, left panel) and the Ba/F3 validation system experiments (Fig. 15) in which shRNA-p16-1 was indicated as the most efficient shRNA. Finally, knockdown efficiencies of the p16 siRNAs were tested on endogenous p16 expression in the MOHITO cell line. We observed the best knockdown for siRNA-p16-3 (Fig. 18B, right panel), as also observed in the Ba/F3 validation system (Fig. 17), while the knockdowns for the other siRNAs were slightly different from those observed in the validation system (Fig. 18B, right panel). Thus overall, the knockdown effects observed for the endogenous transcripts correlated very well to the knockdown measured with the Ba/F3 validation system.

DISCUSSION

Since the discovery of RNAi, gene silencing has been used with increasing interest in both research and therapeutic applications. Over the last years, many algorithms for shRNA and siRNA design have been developed, all based on empirical rules. While these algorithms assist in the selection of RNAi molecules, none of the currently available algorithms can predict the efficiency of siRNAs and shRNAs with high accuracy[143,144]. Here we describe the development and evaluation of a cell-based validation system to complement classical design algorithms to make better choices for effective gene silencing by means of RNAi.

The system we describe here is based on the transforming properties of a truncated form of the PDGFRα kinase (tPDGFRα) in Ba/F3 cells. Ba/F3 cells expressing tPDGFRα proliferate fast (doubling time less than 24 hours) and are completely dependent on the presence of tPDGFRα for their proliferation and survival. We hypothesized that by putting the gene of interest on the same transcript as *tPDGFRα*, we could generate a sensor for efficiency of knockdown of the gene of interest. Effective shRNAs and siRNAs, introduced by transduction or electroporation respectively, target the gene of interest that results in downregulation of the tPDGFRα fusion protein. Loss of tPDGFRα will block proliferation and survival of the Ba/F3 cells, which is an easy read-out to measure in these rapidly proliferating cells.

Our results show that this is indeed possible. We tested the system by cloning the coding sequences of mouse *Cdkn2a* (*p16*) or mouse *Pten* on the *tPDGFRα* transcript (Fig. 14A). Combining two RNA fragments in a transcript may alter the RNA secondary structure, which can affect binding of the RNAi molecules to the target RNA sequence. For this reason we tested three different variants for each gene of interest. We generated (1) p16-tPDGFRα or Pten-tPDGFRα fusion transcripts that encode p16-tPDGFRα or Pten-tPDGFRα fusion proteins, (2) tPDGFRα-p16 or tPDGFRα-Pten fusion transcripts that encode tPDGFRα-p16 or tPDGFRα-Pten fusion proteins, or (3) tPDGFRα*-p16 or tPDGFRα*-Pten fusion transcripts that will express only the tPDGFRα protein, since a stop codon is present at the end of the coding sequence of *tPDGFRα* and the *p16* or *Pten* sequences are thus present in the 3' UTR of this transcript. The documented knockdown effects on protein level and on proliferation of the cells were much more pronounced in the first variant with the p16-tPDGFRα and Pten-tPDGFRα fusion proteins (Fig. 16). This could be the consequence of structural characteristics of the fusion RNAs and could potentially also be

explained by an incomplete degradation of the transcripts when the RNAi molecules are targeting the 3' region of the transcript. In contrast, when the target gene is cloned at the 5' end, a single cut of the transcript will directly lead to a disconnection between the ATG start codon and the PDGFRα reading frame and a direct loss of the fusion protein. We therefore recommend using the set up where the gene of interest is cloned upstream of the oncogenic kinase for future experiments.

We realize that our screening method is an artificial system and therefore has a few disadvantages. It is possible that RNAi molecules with a good knockdown efficiency in the system will not reach this level of knockdown when targeting the endogenous transcripts. Nevertheless, in this study we observed a good correlation between the knockdown efficiency of p16 and Pten obtained in the screening system and the knockdown of endogenous p16 and Pten in mouse cell lines.

In this study we showed that our validation system is a reliable system that is able to identify efficient shRNAs and siRNAs. From three tested shRNAs directed against *p16*, only one shRNA was identified as a shRNA with strong RNAi effect, and this shRNA was confirmed to function well to downregulate the expression of the endogenous *p16* transcript in MOHITO cells. Similarly, a good correlation between the results in the Ba/F3 validation system and knockdown effects on the endogenous gene was observed for *Pten* as well. The validation system detected clear differences between different siRNAs or shRNAs, and a strong correlation was documented with effects on the endogenous transcripts. These data support the Ba/F3 validation system as a potent and easy to use system to select siRNAs or shRNAs with a good knockdown effect.

Our system has a few important differences compared to experimental validation systems described in the past. In those systems a reporter assay is used where a target gene is fused to a fluorescent or enzymatic reporter gene, like EGFP or luciferase respectively. Reduction in the fluorescent or enzymatic activity is observed when effective shRNAs or siRNAs target the gene of interest[147-153,157]. The read-out of our system is the reduction in cell growth due to downregulation of the tPDGFRα protein kinase fused to the target gene. Looking at the direct cellular effects might have some advantages, since protein levels in artificial systems may not always correlate with the cellular effects.

A major challenge for many validation systems is the low transfection or transduction efficiencies, that make it difficult to interpret and compare results of the different shRNAs and siRNAs. A solution to overcome this problem is to include a reference gene[146,147,150-152], but this makes the cloning experiments and the interpretation of results more complex. In our system, siRNA and shRNA delivery is not a major problem: transfection of siRNAs occurs through electroporation, which is highly efficient in the Ba/F3 suspension cell line. It was also not necessary to correct for differences in transduction efficiency, since we could perfectly measure the transduced cells only. All shRNA vectors also contained the *GFP* gene, so that we could gate on GFP positive cells for the shRNA experiments. So even with low transduction efficiency, we were able to observe differences between the different shRNAs. In addition, another advantage of using GFP labeled cells, instead of measuring luciferase activity, is the possibility of measuring several consecutive time points (over several days) with the same set of cells and the direct detection of fluorescence[150].

A second advantage of the Ba/F3 cell system is that the cells can be made independent from the transcripts of interest (here the *tPDGFRα* fusion transcript) by the addition of IL-3. IL-3 is the ligand for the IL-3 receptor, which is expressed by the Ba/F3 cells and which can be used to drive the proliferation and the survival of the cells. In this way, addition of IL-3 will make the cells independent from the *tPDGFRα* fusion transcript, which can be used to investigate if the inhibitory effect observed by the RNAi molecules is due to a specific effect on the *tPDGFRα* fusion transcript or rather due to a non-specific effect. It is plausible that some shRNAs or siRNAs could target critical transcripts in the cell and in this way interfere with the proliferation of the Ba/F3 cells. This would initially cause a false positive read-out in this system. However, when experiments are performed in the absence and also in the presence of IL-3, these false positive results can be detected easily. Finally, it could also be that the shRNA or siRNA target the PDGFRα part of the transcript, rather than the part of the gene of interest. Again, this can be easily detected by using the cells expressing the myc-tPDGFRα fusion, as these cells should not be inhibited by shRNAs or siRNAs specifically targeting the gene of interest.

We can conclude that our validation system is an easy and reliable system to distinguish effective and non-effective shRNAs and siRNAs, and that this system can be used for the selection of the siRNAs and shRNAs with the appropriate knockdown characteristics.

2 Study of PTPRD as a candidate tumor suppressor in acute lymphoblastic leukemia and melanoma

The obtained results of the role of PTPRD as a possible tumor suppressor in melanoma are in preparation for submission.

INTRODUCTION

The receptor tyrosine phosphatase gene *PTPRD* has been described as a tumor suppressor gene because it is frequently mutated, deleted or methylated (promotor methylation) in several types of cancer such as melanoma, GBM, neuroblastoma, lung and head and neck cancer[66,68-70,103,106-108]. Despite the fact that PTPRD is recurrently targeted in various tumors, functional confirmation that PTPRD is a bona fide tumor suppressor protein remains scarce. In the publications of Veeriah et al., Solomon et al. and Meehan et al. it has been shown that reconstitution of PTPRD expression in GBM, melanoma and neuroblastoma cell lines resulted in the suppression of the cell growth and the stimulation of apoptosis. In line, shRNA-mediated knockdown of PTPRD caused an increase in cell growth. Transduction of PTPRD deficient cell lines with PTPRD mutants resulted in a reduction of growth inhibition compared to wild type PTPRD[69,70,111]. Instead, Clark et al. could not confirm these results, since they observed no difference in the amount of colony formation in GBM and neuroblastoma cell lines after reconstitution of PTPRD[158]. Dephosphorylation of STAT3 by PTPRD has been associated with the effects of PTPRD on cell proliferation[70]. In neuroblastoma cell lines PTPRD dephosphorylates AURKA, which is a cell cycle regulated kinase involved in microtubule formation and stabilization of the MYCN oncogene[111]. These genomic and functional data suggest that PTPRD could act as a tumor suppressor gene in several tissues. However, the exact expression pattern of PTPRD is unknown, and the function of PTPRD together with the signaling pathways controlled by this negative regulator are poorly understood.

Since PTPRD seems to be an important tumor suppressor in several solid cancers, we aimed to study in a first part the role of PTPRD as a tumor suppressor in the pathogenesis of ALL. Therefore, we analyzed gene expression, CNVs and mutation profiles of PTPRD in B-ALL and T-ALL cases.

In a second part, we investigated the role of PTPRD in the development of melanoma since PTPRD was described to be frequently mutated (in 12% of the cases) in melanoma[69]. Because of the lack of functional and expression data, it is not clear whether the described mutations are real inactivating mutations or rather passenger mutations. Since the occurrence of passenger mutations in melanoma is high due to UV exposure, it is important to rule out possible passenger mutations. Therefore, we performed expression analysis in melanoma samples and in melanocytes, the cell of origin, to determine the expression levels and its correlation with CNV.

The function of PTPRD and the signaling pathways controlled by this negative regulator are poorly understood. Therefore, we performed some functional experiments in 293T cells to identify downstream signaling proteins and to investigate the difference in enzymatic activity between PTPRD mutants and wild type.

RESULTS

2.1 Loss of PTPRD does not contribute to the development of ALL

The high frequency of PTPRD deletions in ALL correlates poorly with the gene expression levels

Array-CGH data were analyzed with the Agilent Genomic Workbench and Array Studio software to define the percentage of CNV of the *PTPRD* gene. To determine the percentage of *PTPRD* loss in T-ALL patients we analyzed the array-CGH dataset of Gutierrez (42 samples)[159], the array-CGH dataset of Maser (26 samples)[160] and our array-CGH dataset (47 samples). In total, *PTPRD* was heterozygous deleted in 20 out of these 115 T-ALL patient samples, which accounted for a high deletion frequency of 17,4%. It is remarkable that in all these cases the *PTPRD* deletions included the *CDKN2A* locus located 11 Mb downstream of *PTPRD* (Fig. 19A). The tumor suppressor, CDKN2A, is an important regulator of the cell cycle, which is frequently lost in many types of cancer, also in ALL. This raises the question in which extend loss of PTPRD is involved in T-ALL development next to the loss of CDKN2A. The *PTPRD* deletions can be passenger deletions because of the simultaneous loss with *CDKN2A*. Next, we performed expression analysis of our T-ALL sample to define the expression levels of PTPRD. The heatmap of our RNAseq data showed some variation in the PTPRD expression among the T-ALL samples (Fig. 19B).

For the study of *PTPRD* loss in B-ALL patients, we analyzed the dataset of Russell (10 samples)[161] and Kuiper (34 paired diagnosis and relapse samples)[162]. In these B-ALL patients *PTPRD* was heterozygous deleted in 10 out of 44 samples, which is a frequency of 22,7%. Of the nine patients with a *PTPRD* deletion observed in the dataset of Kuiper, four of the patients (ID 5, 11, 15 and 24) contained the *PTPRD* deletion in their diagnosis and relapse sample, three patients (ID 2, 9 and 10) had the *PTPRD* deletion in their diagnosis sample and two patients (ID 13 and 28) had *PTPRD* deleted in only their relapse sample. In six B-ALL samples with loss of *PTPRD*, the deletions included also the *CDKN2A* locus (Fig. 19C). To investigate whether the *PTPRD* deletions results in a reduction of the PTPRD expression level, we performed qRT-PCR and qPCR to define respectively the PTPRD expression levels and CNV in 13 B-ALL samples received from the group of dr. Kuiper (The Radboud University Nijmegen, The Netherlands). Two primer pairs located in exon 14 and 43 of *PTPRD* were used to determine the CVN. The T-ALL cell line, HSB-2, was used as a positive control for the primers used in the q(RT)-PCR. Although it is difficult to define the normal expression level of PTPRD in B-ALL samples with no loss of the *PTPRD* copy number, it seems that there is no correlation between the expression levels and the CNV. From the 5 B-ALL samples (IV-172, IV-149, IV-143, IV-55 and IV-99) with no expression of PTPRD only the IV-99 had a heterozygous deletion of *PTPRD*. For the 3 other samples the CNV was normal and for 1 sample the gDNA was not available to define the CNV. Further, there are four B-ALL samples (IV-140, IV-139, IV-99 and IV-92) with a heterozygous loss of *PTPRD,* which do also have a homozygous loss of *CDKN2A* (Fig. 19D). Although *PTPRD* and *CDKN2A* are both located on the p-arm of chromosome 9, it is not clear from our qPCR data whether the loss of 1 copy of *PTPRD* occurred together with loss of *CDKN2A*, since we do not have the information about the copy number of the 11 Mb between *PTPRD* and *CDKN2A*. Instead, the array-CGH data from dr. Kuiper showed a complete deletion of 9p in the IV-99 and IV-92 samples and mosaic deletions in 9p for the IV-140 and IV-139 samples. Because of the high frequency of simultaneous loss of *PTPRD* and *CDKN2A* in B-ALL and the

array-CGH data of dr. Kuiper, we can assume that both genes are lost together because of the occurrence of bigger deletions in the p-arm of chromosome 9. Although the IV-140, IV-139 and IV-92 lost one copy of *PTPRD*, their mRNA level was not particular lower than the mRNA level in the samples with a normal CNV. One sample (IV-178) had an extremely higher mRNA level compared to the others although no increase of CNV was observed (Fig. 19D). Since our qPCR primers were located in exon 14 and 43, we could not detect smaller deletions in *PTPRD* outside these regions, which could affect the expression levels as well. Neither can we exclude that the expression levels are influenced by other mechanism than CNV. At least we would expect a reduction in expression level, when the CNV is lower, which is not the case in this B-ALL set. That is why we question the contribution of the loss of PTPRD in the development of ALL, although the percentage of *PTPRD* deletions is high.

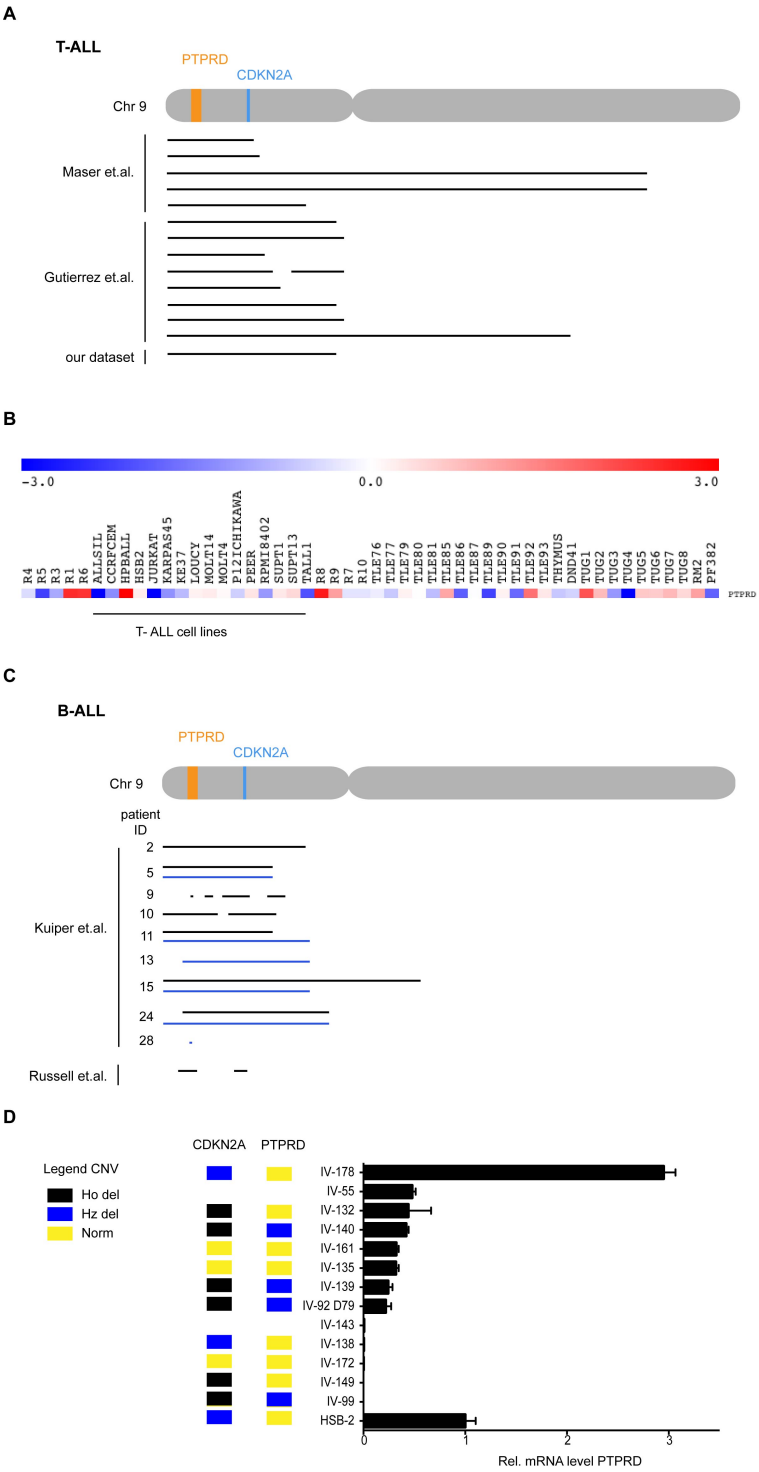


Figure 19. Expression level and copy number variation of PTPRD in T-ALL and B-ALL samples (A) Regions of *PTPRD* deletions on chromosome 9, observed in three array-CGH datasets of human T-ALL patient samples, are indicated with a horizontal line. In all cases the deleted regions included the *PTPRD* and *CDKN2A* locus. (B) Heatmap of RNAseq data visualizes the variation in PTPRD expression among T-ALL patient samples and cell lines. Red indicates a high expression and blue a low expression. (C) Regions of *PTPRD* deletions on chromosome 9, observed in two array-CGH datasets of human B-ALL patient samples, are indicated with a horizontal line. In 6 out of the 10 B-ALL cases *PTPRD* was deleted together with *CDKN2A* in a bigger part. In the three other cases *PTPRD* and *CDKN2A* were lost in two different parts. One patient contained only a smaller deletion in the *PTPRD* locus. Deletions in diagnosis samples are indicated with a black line and relapse samples with a blue line. (D) Variation of PTPRD mRNA level in B-ALL patient samples did not correlate with the copy number of *PTPRD*. The four patient samples with heterozygous loss of *PTPRD* had a homozygous deletion of *CDKN2A*. A normal copy number is indicated with a yellow box, a heterozygous deletion with a blue box and a homozygous deletion with a black box. The T-ALL cell line HSB-2 was used as a positive control for the primers used in the qPCR and qRT-PCR experiments.

Point mutations in *PTPRD* are rare in ALL

When we assume that *PTPRD* is a tumor suppressor gene, we would expect to detect inactivating mutations in the patients with no deletions of *PTPRD* and in the patients with loss of only one copy. Therefore, we identified the amount of *PTPRD* mutations in two independent sets of ALL samples received from the group of prof. Soulier (University Paris-Diderot, France) (80 samples) and from the group of prof. Harrison (Newcastle University, UK) (75 samples) with the haloplex target enrichment technique and in our own T-ALL sample set with exome sequencing (48 samples). Exome sequencing is a technique of high-throughput sequencing of the coding regions of the genome, whereas Haloplex is a high-throughput technique, whereby selected genomics regions of interest are enriched and sequenced. We analyzed these datasets with the NextGENe software of Softgenetics. As parameters, we set the depth coverage at a minimum of 20 reads and the variant allele frequency needed to be higher than 20%. The variant allele frequency indicates how frequent the variant is present according to the total amount of reads. Variants present in a single nucleotide polymorphism database (dbSNP) are indicated in the software.

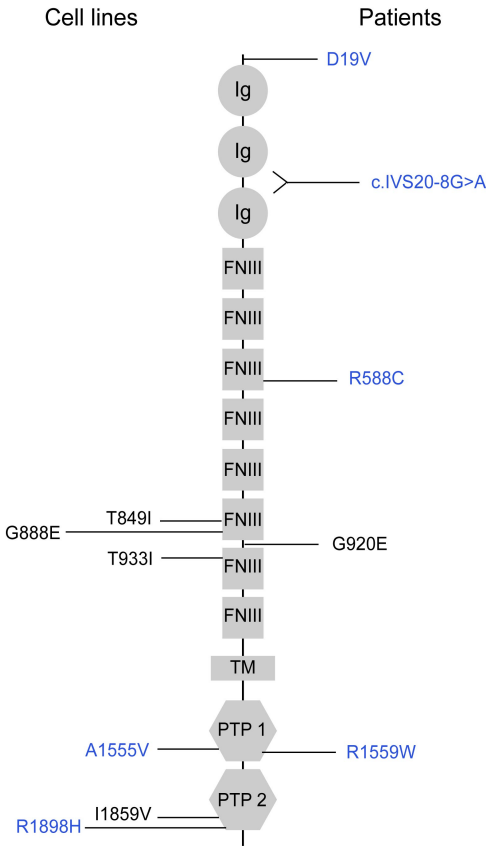


Figure 20. Location of the PTPRD single nucleotide variations and single nucleotide polymorphisms observed in T-ALL cell lines and patient samples The location of the PTPRD single nucleotide variations (SNVs), indicated in black, and single nucleotide polymorphisms (SNPs), indicated in blue, observed in the T-ALL cell lines (*left*) and in the T-ALL patient samples (*right*). The SNPs are marked in blue and the SNVs in black.

The sequence analysis revealed only one single nucleotide variant (SNV) and four single nucleotide polymorphisms (SNPs) in five out of the 203 T-ALL patients. A SNP is a variation in the DNA sequence, whereby a single nucleotide differs between individuals. In contrast to a SNV, a SNP is assumed to not cause a disorder. These variants were validated with sanger sequencing. One of the four SNP (c.IVS20-8G>A) appeared in our exome sequencing dataset and was described in the 1000 genomes project (Table 5). This SNP was located in the extracellular part of PTPRD nearby the splice site region before exon 20 (Fig. 20). According to the Variant Effect Predictor (VEP) of Ensembl this SNP would not have a functional consequence. The three other SNP (D19V, R588C and R1559W), present in the dbSNP138, were found in the haloplex target enrichment dataset of prof. Soulier (Table 5). Two of these SNP (D19V and R588C) were located in the extracellular domain of PTPRD and the R1559W SNP was located in the first catalytic domain of PTPRD. The only SNV G920E appeared in the dataset of Soulier and was located in the extracellular domain of PTPRD (Fig. 20). With the SIFT and Polyphen prediction tools, we can predict whether these amino acid substitutions are affecting protein structure and function. According to the Polyphen tool the R588C and R1559W SNPs and the G920E SNV are probably damaging and the D19V SNP is benign. The SIFT tool indicates the D19V and R588C SNPs as tolerated, but the R1559W as deleterious (Table 5). These data indicate that PTPRD mutations are rare in T-ALL and it remains to be determined if the SNVs detected in this set of 203 T-ALL cases have functional consequences.

Table 5. Single nucleotide variations and polymorphisms in T-ALL samples

Variant	SNV/SNP	SNP database	VEP	SIFT	Polyphen	Sequencing database
c.IVS20-7C>T	SNP	1000 genomes project	No consequence	/	/	Cools
D19V	SNP	dbSNP138	/	Tolerated	Benign	Soulier
R588C	SNP	dbSNP138	/	Tolerated	Probably damaging	Soulier
G920E	SNV	No	/	/	Probably damaging	Soulier
R1559W	SNP	dbSNP138	/	Deleterious	Probably damaging	Soulier

PTPRD alterations and expression levels in T-ALL cell lines

Western blot analysis and qRT-PCR showed some variation in PTPRD expression levels among our 18 T-ALL cell lines (Fig. 19B and 21). The CNV of the *PTPRD* gene are determined with either array-CGH (indicated in black) or qPCR (indicated in blue) with primers located in exon 14 and 43. The variation in expression levels could only partially be explained by CNV. The reduced expression of PTPRD in the CCRF-CEM and P12-ICHIKAWA cell lines can be the consequence of the heterozygous loss of the *PTPRD* gene (Fig. 21). The P12-ICHIKAWA harbors a mutation in the extracellular part of the protein in the remaining copy (Fig. 20). The MHH T-ALL cell line had a homozygous deletion of *PTPRD* and showed no expression. The PTPRD gene was duplicated in the HPB-ALL and the PEER cell line, but only the HPB-ALL had a high expression (Fig. 21). Besides P12-ICHIKAWA, four other cell lines contained a mutation in PTPRD. The P12-ICHIKAWA, JURKAT and the HSB-2 cell line had a mutation in the extracellular part and KARPAS-45 and SUP-T1 had a mutation in the catalytic domain of the protein. With a mutation rate of 28%, the presence of mutation is much higher in cell lines compared to patient sample. This can be explained with the increasing occurrence of genomic alteration by keeping cell lines in culture.

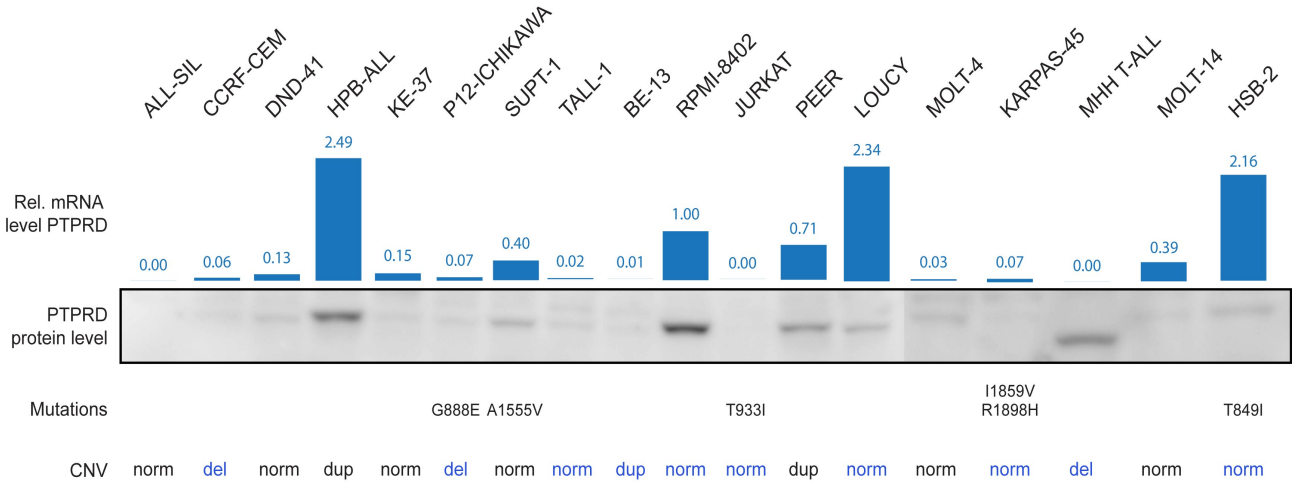


Figure 21. Expression level and genetic alterations of PTPRD in T-ALL cell lines The variation in PTPRD expression between the 18 T-ALL cell lines was observed at the mRNA and protein level. This variation in expression level was only partially explained by a change in the copy number of *PTPRD*. The CNV was determined by array-CGH, indicated in black, or qPCR, indicated in blue. Four out of the 18 T-ALL cell lines had one or two point mutations in PTPRD.

Possible role of PTPRD in hematopoiesis

Although the relevance of loss of PTPRD in ALL is not confirmed, it seems that PTPRD plays a role in the hematopoiesis. First indication is a higher expression of PTPRD in hematopoietic stem cells and early progenitors compared to more differentiated cells after analysis of the human hematopoietic cell expression data of Novershtern et al. (Fig. 22A). Novershtern et al. purified 38 distinct populations of human hematopoietic cells from umbilical cord and peripheral blood. After isolation of mRNA from each cell type, they performed expression profiles using Affymetrix microarrays[163]. The PTPRD gene expression profiles in the distinct hematopoietic cells were analyzed using the online available webtool of the Broad Institute (http://www.broadinstitute.org/dmap/home). Second, morphological examination of the spleen showed severe atrophy with reduction of the white and red pulpa in 16 weeks old *Ptprd* knockout mice housed in a specific pathogen free (SPF) facility. In 7-8 months old mice, housed in conventional facility, extramedullar hematopoiesis was observed in the spleen compared to control, because of a high amount of myeloid progenitors (Fig. 22B). These data indicate an important role of PTPRD in the hematopoiesis.

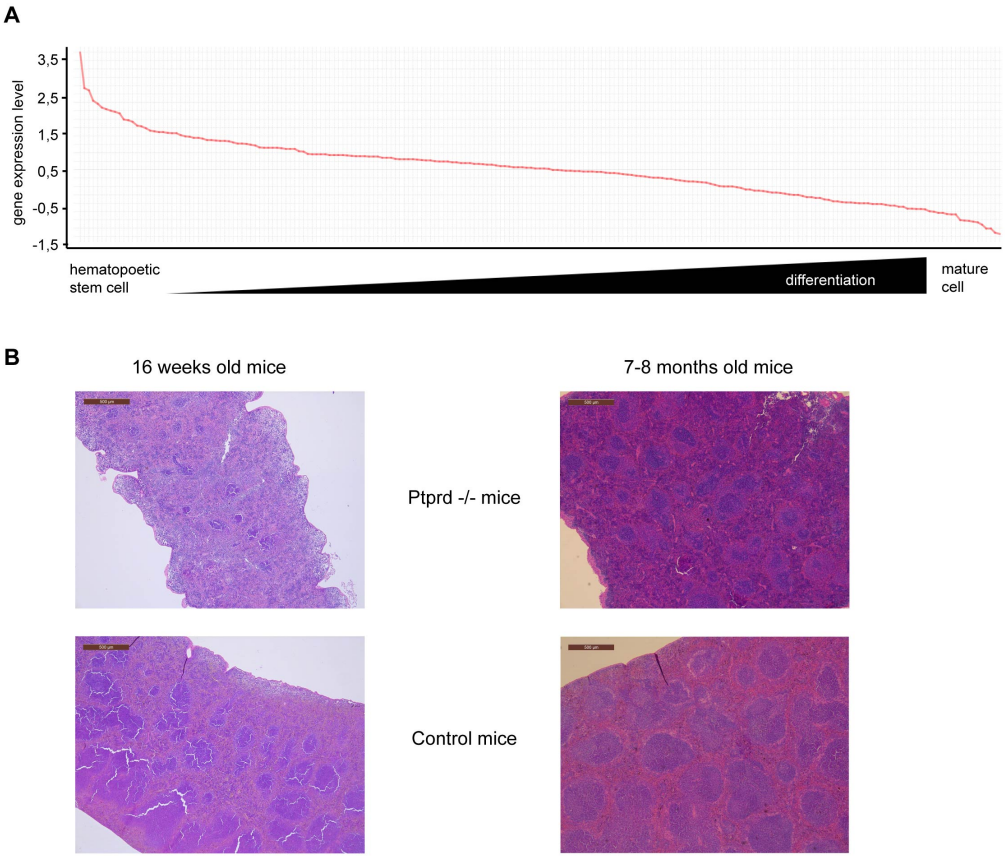


Figure 22. Higher PTPRD expression in undifferentiated hematopoietic cells and spleen defects in *Ptprd* knockout mice indicates a possible role in hematopoiesis (A) PTPRD expression levels drops along the differentiation process of the hematopoietic cells. (B) The 16 weeks old *Ptprd* knockout SPF mice had severe spleen atrophy with reduction of the white and red pulpa (*left*). Extramedullar hematopoiesis in the spleen of 7-8 months old conventional *Ptprd* knockout mice was observed because of a high amount of myeloid progenitors present in the spleen (*right*).

2.2 Loss of PTPRD does not contribute to the development of melanoma

In literature is described that PTPRD is mutated in 12% of the melanoma cases[69,70]. Due to the lack of expression analysis and extensive functional experiments, it is not clear whether these mutations are real inactivating mutations or passenger mutations. Since the induction of passenger mutations in melanoma is high because of UV exposure, we aimed to study the role of PTPRD as a possible tumor suppressor in more detail. We started with performing mutation analysis to confirm the high mutation rate of PTPRD in melanoma. Next, we analyzed expression levels and CNV of PTPRD in melanoma.

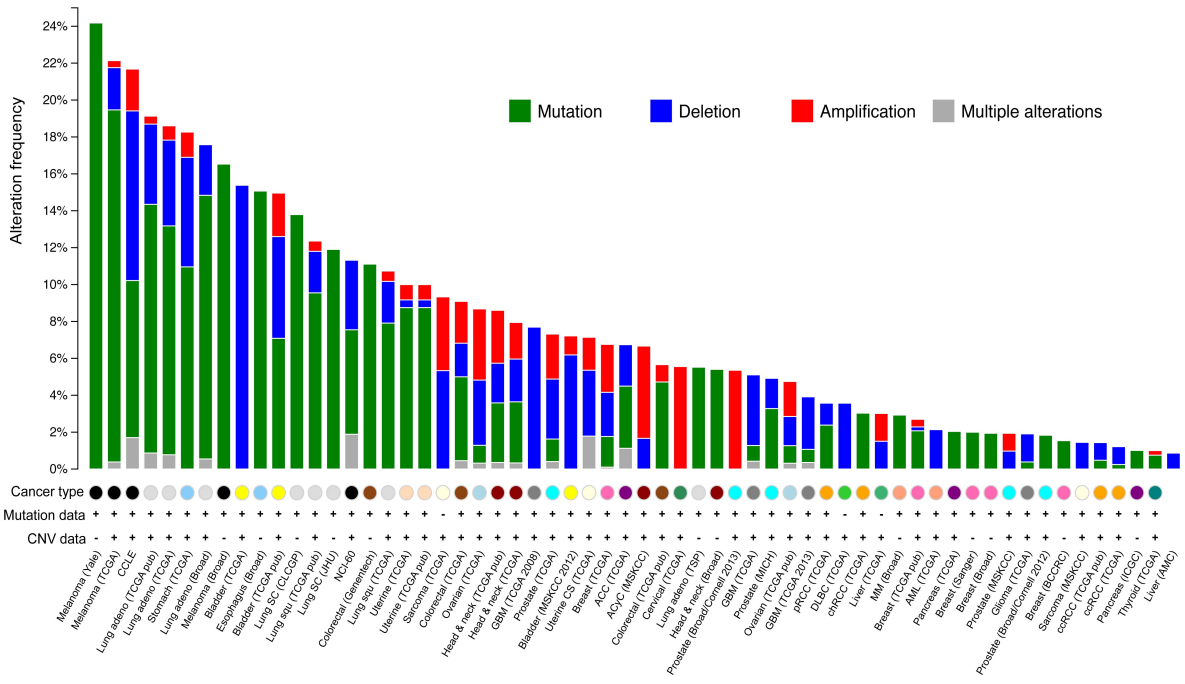
PTPRD is highly mutated in melanoma

To determine the spectrum of *PTPRD* mutations in melanoma, we analyzed the exome sequencing datasets described by Hodis (121 samples)[164], Krauthammer (91 samples)[107] and The Cancer Genome Atlas (TCGA) initiative (262 samples). Our analysis revealed that 92 out of 474 patients had a missense (94,4%), nonsense (4,2%) or a splice mutation (1,4%) in PTPRD. This high mutation rate of 19,4% exceeded the previously described frequency of *PTPRD* mutations in melanoma. Compared to mutation frequency of *PTPRD* in other types of cancer, the frequency is the highest in melanoma (Fig. 23A). These mutations were found to be spread all over the gene (Fig. 23B). And more mutations were found in the introns (69,3%) compared to the exons (30,7%) of the exome sequencing dataset from Hodis et al. The high frequency of mutations in melanoma can be explained by UV exposure of the skin. The UVB radiation in sunlight induces mainly C to T or CC to TT transition by the formation of cyclobutane pyrimidine dimers (CPD). The CDP consists of a squared ring with four carbons arising from the coupling of the C=C double bonds of pyrimidines. These dimers interfere with base pairing during DNA replication, resulting in mutations. The cytosine in the CPD is prone to be deaminated, which induces the C to T transition[165]. In the analyzed datasets 40% of the *PTPRD* mutations contained a C to T transition and 3% a CC to TT transition. Of the 32 *PTPRD* mutations present in the dataset of Krauthammer, 26 were found in sun-exposed melanoma with acral, mucosal or uveal origin. Furthermore, 29 of these 32 mutations contained the C to T or CC to TT transitions, which are typically induced by UV exposure. To determine if the detected mutations in *PTPRD* can be driver oncogenic mutations, we hypothesized that there would be an enrichment of mutations compared to mutations in introns due to selection. This was not the case for the mutations found in *PTPRD*, since 69,3% mutations were located in the introns compared to 30,7% in the exons. Instead, the *BRAF* and *NRAS* oncogenes and the *TP53* tumor suppressor gene, in melanoma contained a higher percentage of mutations in the exons (respectively 90%, 93,8% and 85,2%) compared to the introns (respectively 10%, 6,2% and 14,8%) (Table 6). Taken together, our mutation analyses were a first indication that the mutations in melanoma are passenger mutations.

Table 6. Percentage of mutations located in exons and introns of oncogenes and tumor suppressor in melanoma

Gene	Mutation in exons (%)	Mutation in introns (%)
PTPRD	30,7	69,3
BRAF	90	10
TP53	85,2	14,8
NRAS	93,8	6,2

A



B

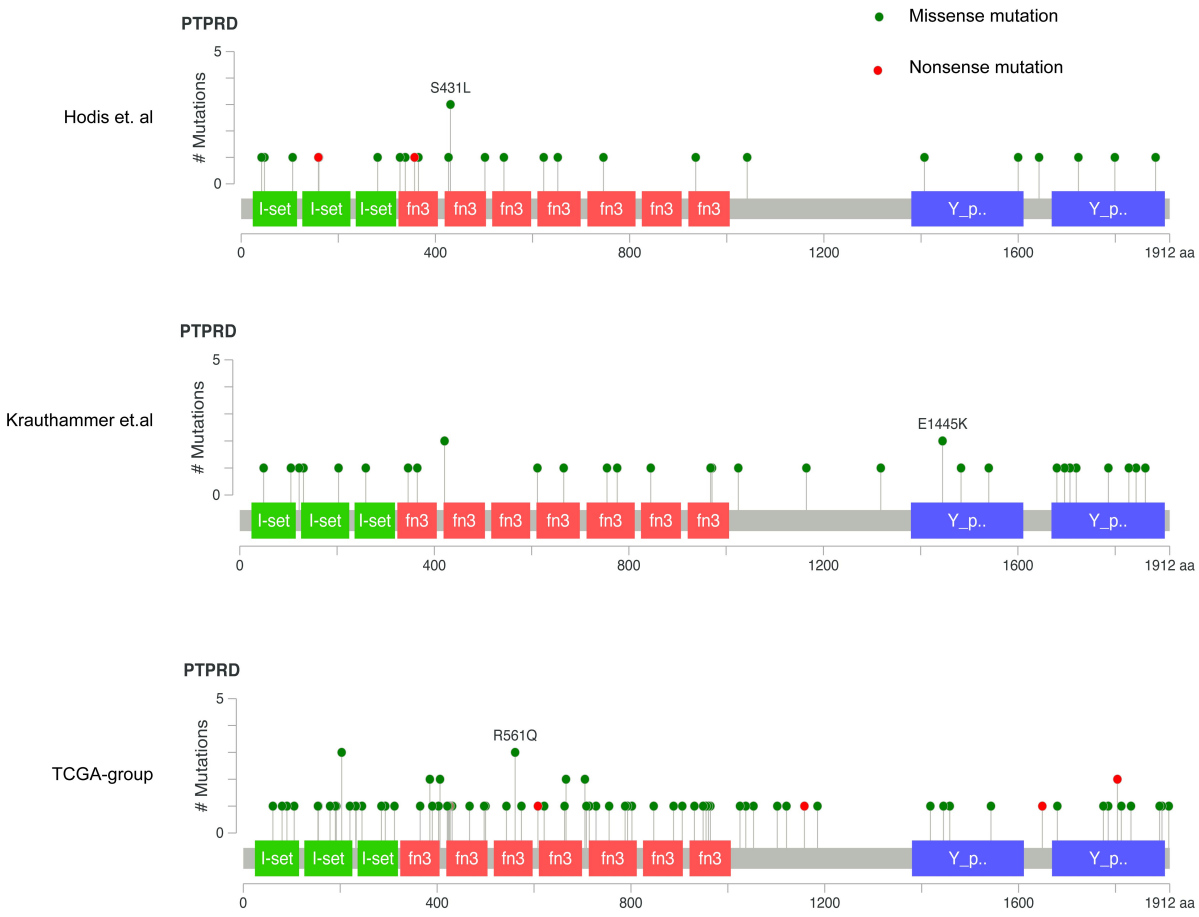


Figure 23. Melanoma counts a high amount of *PTPRD* mutations with a distribution all over the gene (A) According to the cbioportal webtool the mutation rate of *PTPRD* is the highest in melanoma compared to other cancer types. (B) In three different sequencing datasets the mutations were spread all over the *PTPRD* gene and more missense than nonsense mutations were present.

PTPRD is expressed at very low levels in melanoma

To further investigate the possible role of loss-of-function mutations of PTPRD in melanoma development, we performed and analyzed gene expression in melanoma cell lines and patient samples. In five melanoma cell lines there was no PTPRD mRNA or protein detected. Except from a heterozygous deletion of *PTPRD* in the COLO-800 cell line, this absence of PTPRD expression could not be explained by deletion of *PTPRD* (Fig. 24A). Analyses of gene expression profile datasets with Oncomine and Genevestigator showed that PTPRD is expressed at very low levels in melanoma samples. Point mutations are not necessarily affecting the PTPRD expression level, unless nonsense mutations introduce an earlier stop codon. As earlier described, the occurrence of nonsense mutations (4,2%) is not so high in melanoma compared to missense mutations (95,6%). Instead, CNVs have an influence on the protein expression levels, but homozygous deletions were observed in only 7 of the 262 (2,7%) patient samples of the TCGA dataset. Therefore, we analyzed mRNA levels and CNV in 12 mouse melanoma samples and in 57 human melanoma samples by performing qRT-PCR and qPCR. RNA quality was determined with the Bio-analyzer and samples with a RIN value below 5 were excluded from the experiment. The Ct values from the human and mouse melanoma samples clustered around the value of 30, which confirmed the very low expression of PTPRD in melanoma (Fig. 24B). Human melanoma samples with lower Ct values (between 25 and 28) could be assigned due to the lower Ct value of the B2M housekeeping gene or due to the lower percentage of tumor cells in the sample. Human and mouse melanoma tumor samples with a Ct value exceeding 30 for the melanocyte specific markers TYR and DCT were excluded from the analysis. Moreover, the expression level of PTPRD in healthy human melanocytes, the cell of origin of this cancer, was also extremely low (Ct values 30 or more) compared to other tissues like brain in which the functional role of PTPRD is more obvious (Fig. 24C).

To define whether these low expression levels are the consequence of alteration in copy number, we compared the relative mRNA levels with the CNV in half of the human melanoma sample. In 15 samples with a heterozygous deletion of *PTPRD* only 8 samples had a low PTPRD mRNA level. For the samples with a high PTPRD mRNA level the copy number was not increased (Fig. 24D). Whether the increased mRNA levels of the samples in this graph indicate an effective increase in PTPRD expression is hard to conclude from this experiment, since the samples are compared against the low PTPRD expression in the melanocyte sample. We can conclude that the expression levels of PTPRD did not correlate with its CNV, as you would expect when tumor suppressor are lost in cancer. As in ALL, the loss of one copy of *PTPRD* could be assigned to the loss of the CDKN2A tumor suppressor, both located on the p-arm of chromosome 9. Except from 1 sample, all human melanoma samples with a deletion of *PTPRD* did also contain a deletion of *CDKN2A*. From our data it is not clear whether loss of *PTPRD* occurred together with loss of *CDKN2A*, due to the lack of the information about the CNV between the *PTPRD* and *CDKN2A* genes. We would expect that deletion of a tumor suppressor gene would result in an obvious reduction of its gene expression. Indeed, loss of the well-known tumor suppressor genes, *PTEN* and *CDKN2A*, in melanoma had indeed a much higher correlation between their expression and CNV, compared to PTPRD (Fig. 24E). Although there is not enough evidence from our study that PTPRD function as a tumor suppressor in melanoma, that doesn't rule out the possible tumor suppressive role of PTPRD in other cancer types. For the cancer types with described *PTPRD* deletions, such as GBM, lung cancer and head and neck cancers, we generated correlation plots for PTPRD gene expression and CNV of the melanoma dataset of the TCGA-group using the cbioportal online

webtool. In GBM, lung cancer and head and neck cancer a small reduction in the expression level of PTPRD is observed when the gene is deleted (Fig. 25). A potential tumor suppressive role of PTPRD in these cancer types should be studied in more detail.

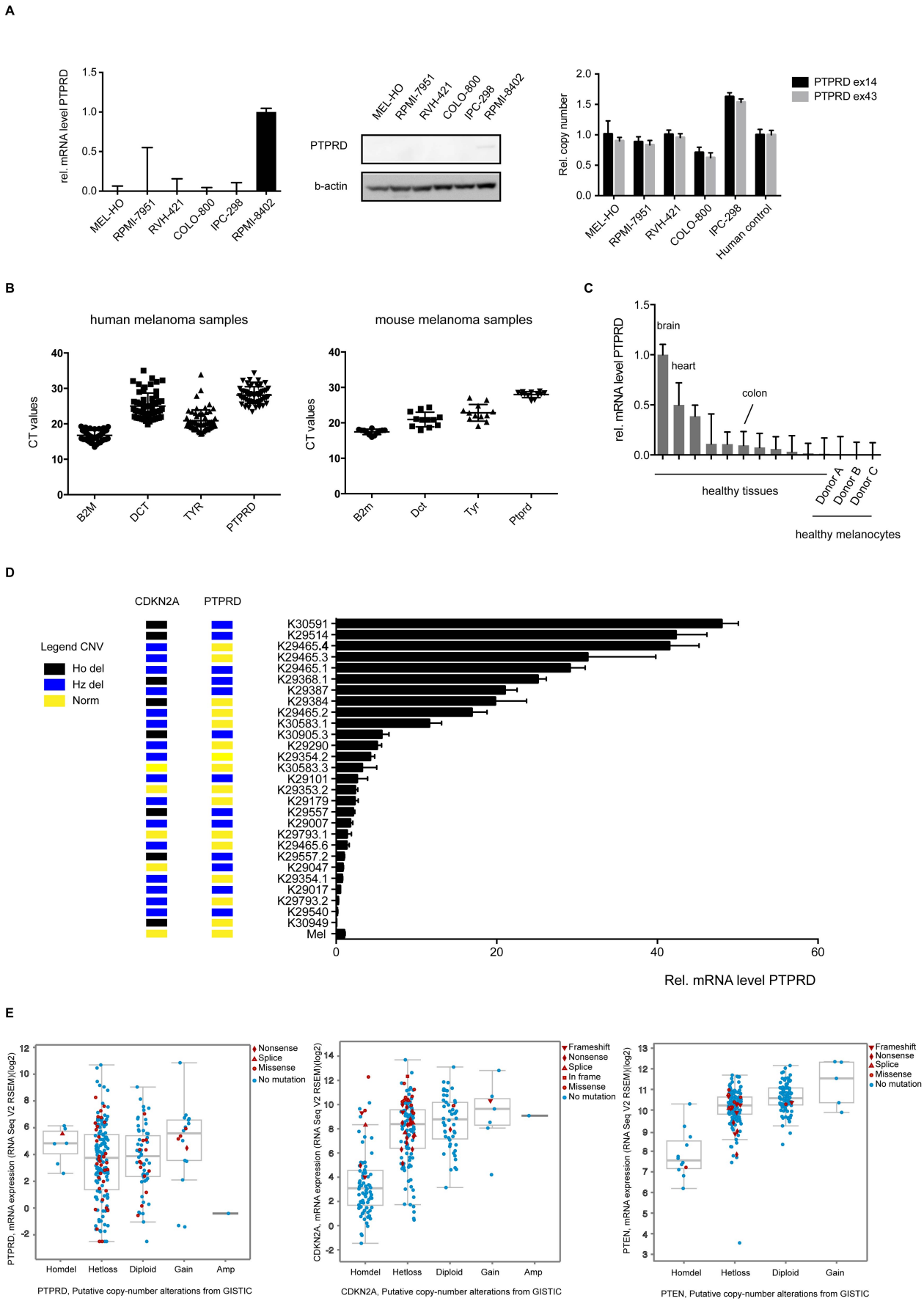


Figure 24 (opposite page). Expression level and copy number variation of PTPRD in melanoma cell lines and samples (A) The mRNA level (*left*) and protein level (*middle*) showed no PTPRD expression in the five human melanoma cell lines. Except from a heterozygous deletion of *PTPRD* in the COLO-800 cell line, this absence of PTPRD expression could not be explained by a change in copy number variation (*right*). (B) The PTPRD expression in human (*left*) and mouse (*right*) melanoma samples is very low, because the Ct values cluster around 30. Tumor samples with a Ct value of 30 or higher for the melanocyte specific markers TYR and DCT were excluded from the analysis. The human and mouse B2M genes were used as a housekeeping gene to rule out high differences in mRNA starting material. (C) The PTPRD expression in the melanocytes from healthy donors is extremely low compared to other human tissues. (D) Variation of PTPRD mRNA level in melanoma patient samples did not correlate with the copy number of *PTPRD*. Except from one sample, all the patient samples with a heterozygous loss of *PTPRD* had lost at least one copy of *CDKN2A*. A normal copy number is indicated with a yellow box, a heterozygous deletion with a blue box and a homozygous deletion with a black box. The human melanocyte was used as a reference sample in the qPCR and qRT-PCR analysis. (E) Comparison of PTPRD expression level and CNV showed no correlation in the public available dataset of the TCGA-group using the cbioportal web tool. Instead, there is an obvious reduction in the gene expression of the known tumor suppressor genes *PTEN* and *CDKN2A*, when these genes are lost in melanoma.

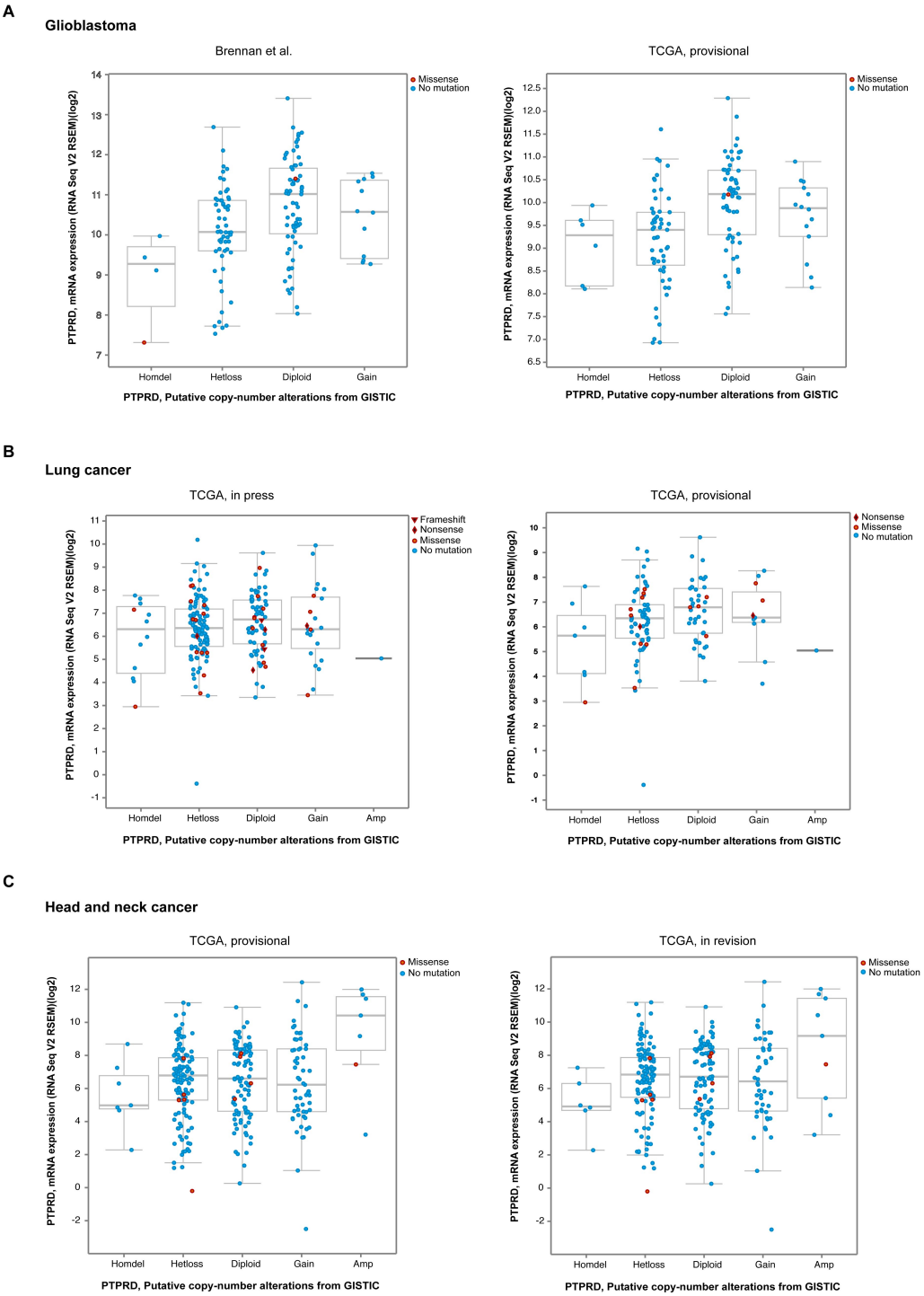


Figure 25. Correlation plots of PTPRD expression levels and copy number variation in glioblastoma multiforme, lung cancer and head and neck cancer The correlation between the PTPRD expression levels and CNV was analyzed in two different dataset of the glioblastoma multiforme (GBM), lung and head and neck cancer types. Deletions of *PTPRD* were previously reported for these types of cancer. There is small reduction in the mRNA level of PTPRD when the gene is deleted in (A) GBM, (B) lung cancer and (C) head and neck cancer.

The STAT1 and STAT3 proteins as substrates of PTPRD in 293T cells

Although it seems that PTPRD is not involved in the development of melanoma and ALL, we confirmed STAT3 as a substrate of PTPRD and identified STAT1 as a yet unknown substrate in 293T cells. We used the 293T cells as model to analyze the phosphatase activity of wild type PTPRD and mutants. The 293T cells were transfected with a ½ dilution serie from 1 to 0 µg DNA of the PTPRD wild type and mutant constructs. The level of substrate dephosphorylation was indicative for their phosphatase activity.

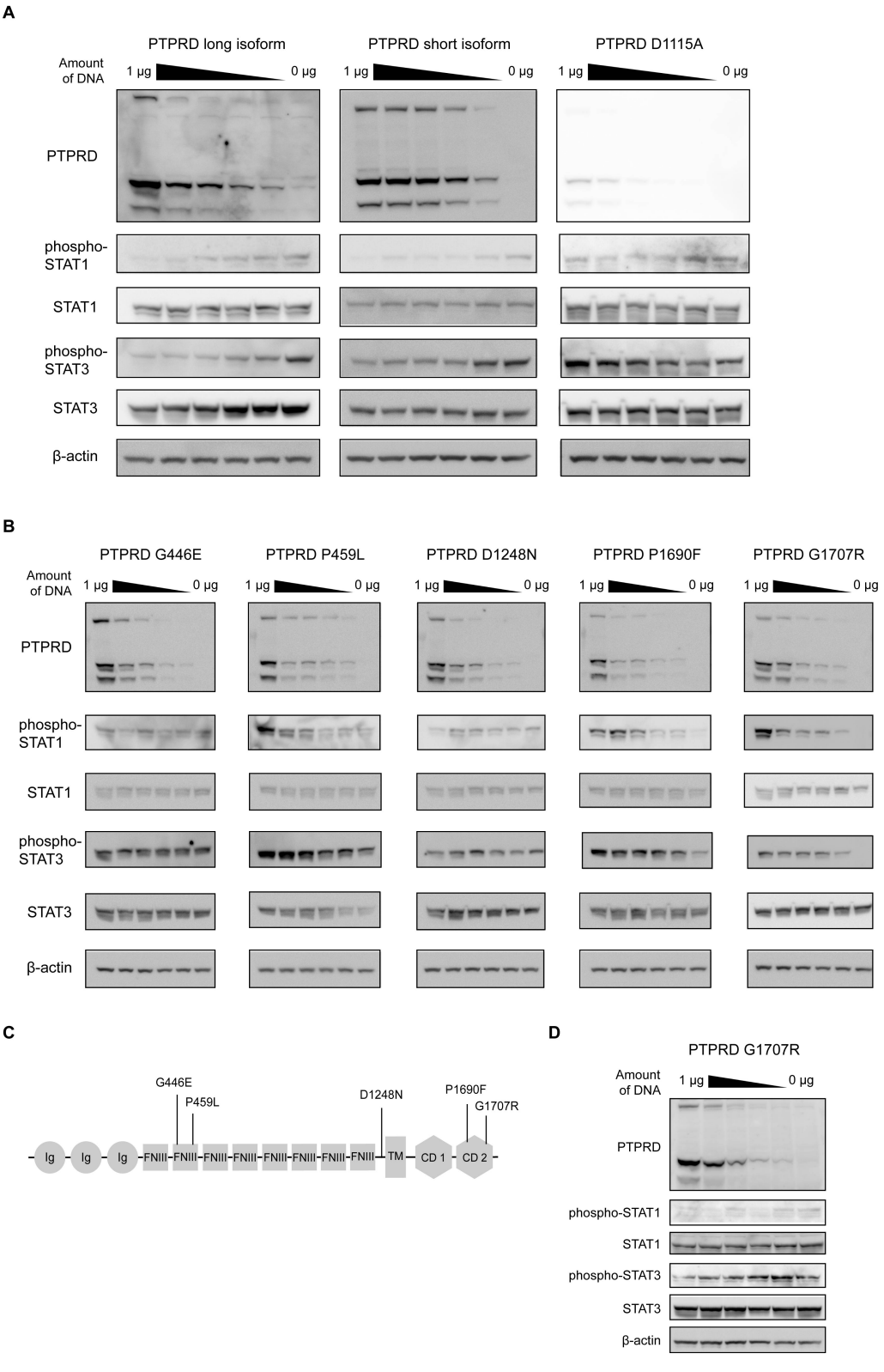


Figure 26 (opposite page). Study of the phosphatase activity of wild type PTPRD and mutants in 293T cells (A) The PTPRD long and short isoform are fully catalytic active, since a higher expression level resulted in a bigger decrease in pSTAT1 and pSTAT3 compared to lower expression levels. Instead, there was no difference in the phosphorylation level of STAT1 and STAT3 among the several expression levels of the catalytically inactive PTPRD D1115A mutant. (B) The PTPRD mutants P459L, P1690F and G1707R seems to act as oncogenes, since higher expression levels of these mutants resulted in a higher pSTAT1 and pSTAT3 level. No difference in the phosphorylation level of STAT1 and STAT3 was observed for the PTPRD mutants G446E and D1248N. (C) Location in the *PTPRD* gene of the five PTPRD mutants identified in melanoma and GBM. (D) The oncogenic effect of the PTPRD G1707R mutant, which was seen in panel B, could not be repeated.

Increasing expression levels of the wild type long and short isoform of PTPRD resulted in a higher dephosphorylation of STAT3 and also STAT1. In contrast the enzymatically inactive protein PTPRD D1115A was no longer able to dephosphorylate its substrates (Fig. 26A). The STAT proteins are well known transcription factors that promote cell proliferation. The PTPRD proteins seem to have a tumor suppressive role, because they are able to dephosphorylate the STAT1 and STAT3 proteins. The effect of the PTPRD point mutations on its phosphatase activity was analyzed by studying the phosphorylation status of STAT1 and STAT3 in 293T cells after transfection with the PTPRD mutants. The five PTPRD mutants were located in the extracellular and intracellular part of PTPRD (Fig. 26B) and were previously identified in melanoma or GBM cases[69]. Remarkably, higher expression levels of the PTPRD P459L, P1690F and G1707R mutants induced a higher phosphorylation of STAT1 and STAT3, suggesting an oncogenic role (Fig. 26C). Unfortunately, we were not able to reproduce this result in 293T cells (Fig. 26D). Other models should be used to study the effect of the mutation on the PTPRD phosphatase activity, since the results from the 293T model is inconclusive.

DISCUSSION

Previous publications report *PTPRD* as a candidate tumor suppressor gene, because the gene is frequently lost in several solid tumors. A possible role of PTPRD as a tumor suppressor in hematological malignancies was not yet described. We observed a high frequency of heterozygous deletions of *PTPRD* in array-CGH datasets of T-ALL and B-ALL cases. Remarkably, almost in all cases the *PTPRD* gene was lost in a bigger part together with the *CDKN2A* gene. This simultaneous loss questions the relevance of PTPRD as a tumor suppressor next to the well-known tumor suppressor CDKN2A. The CDKN2A is a cell cycle regulator, whose losses contribute in many cancer types to development of the malignant cells. Deletion of a tumor suppressor gene would result in a reduction of its expression level. Unfortunately, our expression analysis of PTPRD in the B-ALL samples does not correlate with the CNV analysis of the gene. According to the two-hit hypothesis, loss of only one copy of *PTPRD* can be insufficient to inactivate a tumor suppressor gene[166]. Because *PTPRD* is frequently heterozygous deleted in ALL, we performed a mutations analysis in T-ALL samples. We observed only one SNV and four SNP in the T-ALL samples. In T-ALL cell lines the frequency of PTPRD mutations was higher than in the patient samples, but this can be due to long time culture of the cell lines. Mutations are unlikely to be involved in the inactivation of PTPRD in T-ALL, because of their low frequency. Of course we can't exclude other mechanisms, such as promoter methylation or regulation by miRNAs, which causes the observed variation in PTPRD expression levels in ALL. From our results we have reason to doubt about the tumor suppressive role of PTPRD in ALL, because the reduction in expression level does not correlate with a lower CNV and the high frequency of *PTPRD* deletions can be a consequence of the simultaneous loss of *CDKN2A* due to large deleted regions on Chr9p. Although we couldn't prove the

tumor suppressive role in ALL, we showed first evidence that PTPRD might be involved in the hematopoiesis. The *Ptprd* knockout mice had a severe spleen atrophy and extramedullary hematopoiesis in the spleen. Hematopoiesis that occurs in organs other than the bone marrow is called extramedullary hematopoiesis. Since the extramedullary hematopoiesis was observed in conventional *Ptprd* knockout mice, which are not pathogen free, the extramedullary hematopoiesis can be caused because of an active immune response to pathogens. Therefore, analysis of *Ptprd* knockout mice housed in a SPF facility can obtain more insights. The extramedullary hematopoiesis in the spleen can also occurs when the bone marrow is failing in sustaining the hematopoiesis. For examples, due to myelofibrosis, hematopoietic cells will migrate outside the bone marrow[167]. However, H&E staining of the bone marrow showed no morphological differences with control mice. Another indication for the role of PTPRD in hematopoiesis is the higher PTPRD expression level in hematopoietic stem cells and progenitor cells compared to differentiated cells. The potential role of PTPRD in the hematopoiesis should be investigated in more detail by performing flow cytometry analysis and more morphological analysis of the hematopoietic tissues of *Ptprd* knockout mice in order to confirm the preliminary data and to understand the underlying mechanism of this phenotype.

Besides our study of the tumor suppressive role of PTPRD in ALL, we also investigated its role in melanoma because a high frequency of PTPRD mutations was described in this malignancy. In general the mutation rate in melanoma is high due to sun exposure and therefore also the occurrence of passenger mutations is high. From the literature it is not clear whether the described PTPRD mutations are inactivating or passenger mutations because the biological relevance of these mutations was not studied in great detail. Our expression analyses showed a very low expression of PTPRD in human melanoma cell lines and in human and mouse melanoma samples. Even in the melanocytes, which are the cells of origin of the melanoma, the PTPRD expression is extremely low. Because of low PTPRD expression level in melanoma and melanocytes, its biological function in this malignancy is under question. When UV irradiation induces passenger mutations in the *PTPRD* gene, we would expect at least as many mutations in the introns as in the exons. Mutations in the untranslated regions of the gene will not give a proliferative advantage to the cells, unless it interferes for example with its splicing. Indeed, we observed the double amount of mutations in the introns than in the exons. Compared to known oncogenes, *BRAF* and *NRAS*, and tumor suppressor gene, *TP53*, the amount of mutations in the exons is higher because they provide a proliferative advantage to the cells, which results in their selection. Mostly these mutations are present in more than one patient sample and are therefore called hotspots. For example the BRAF V600E mutant is present in 80% of the melanoma cases. We did not observe any mutation hotspot in the *PTPRD* gene. Instead, the mutations were spread all over the gene, which indicates the more randomized introduction of the passenger mutations in the *PTPRD* gene. However, that doesn't mean that substitutions at several places in the gene could not affect its protein activity or function. The typically UV-induced C to T transitions were also high in the *PTPRD* gene. These observations from our mutational analysis indicate that the *PTPRD* mutations are likely to be passenger mutations. However this high rate of *PTPRD* mutations does not explain the low expression levels in melanoma and even low protein levels can have an important cellular function. If PTPRD is an important tumor suppressor in melanoma, we would expect even lower expression levels when the gene was deleted. In fact our comparison between gene expression and CNV in human and mouse melanoma samples showed no correlation. All together our data indicate that mutations and deletions found in melanoma are unlikely to be relevant for the development of the tumor.

Our functional studies in 293T cells confirmed STAT3 as a downstream signaling protein of the long and short isoform of PTPRD and identified STAT1 as a new downstream target of PTPRD. In contrast to the wild type PTPRD, some PTPRD mutants had an oncogenic effect by increasing the STAT1 and STAT3 phosphorylation. Although we could not repeat this effect in 293T cells, Lui et al. published the same observation with PTPRT mutants in head and neck squamous cell carcinomas. Overexpression of PTPRT proteins with mutations in their catalytic domain in head and neck squamous cell carcinoma resulted in an increased STAT3 phosphorylation. Their computational modeling of the PTPRT mutations showed that these mutations interfere with phosphatase activity and/or substrate interactions. Furthermore almost 50% of the PTPRT mutations were located in the phosphatase domain, supporting the relevance of their interference with the enzymatic activity. In general this group found a significant association between mutations in the receptor PTPs, including PTPRD, and increased pSTAT3 levels[168]. A possible oncogenic effect of the PTPRD mutants by increasing pSTAT3 levels should be further investigated in a relevant tumor model.

2.3 The identification of PTK2B as a downstream target of PTPN2

INTRODUCTION

The Protein Tyrosine Phosphatase, non-receptor type 2 or PTPN2 is an intracellular, non-transmembrane phosphatase, which is highly expressed in hematopoietic cells. The PTPN2 protein is highly expressed in hematopoietic cells and its absence accounts for the defects in hematopoiesis observed in the *PTPN2* knockout mice, which die within 3 to 5 weeks due to immunological dysfunction[117,121]. Studies in T-cell specific deficient mice indicate PTPN2 as a negative regulator of TCR signaling that sets the threshold for TCR-induced naïve T-cell response[122]. The identification of SNP upstream of the PTPN2 locus implicates it role in human autoimmune diseases[169]. Furthermore, PTPN2 has also been implicated in cancer development and is considered as a tumor suppressor in T-ALL, Hodgkin lymphoma and T-cell non-hodgkin lymphoma[43,44,129]. However, the downstream pathways, regulated by PTPN2 in leukemia development and progression, are not yet completely understood.

In T-ALL, focal deletions of *PTPN2* are observed in about 6% cases, and it was shown that loss of PTPN2 leads to an increased signaling downstream of the IL-2 and IL-7 cytokine receptors in leukemic cell lines. These observations suggest that loss of PTPN2 enhances the response of T-ALL cells to these cytokines and provides the leukemia cells with a growth advantage[43]. In addition, it was observed that loss of PTPN2 frequently co-occurs with the NUP214-ABL1 fusion or with mutation of JAK1. Indeed, overexpression of these oncogenic kinases (NUP214-ABL1 or mutant forms of the JAK1 kinase) together with knockdown of PTPN2 accelerated the transformation of leukemic cells *in vitro*, demonstrating cooperation between these oncogenic lesions[43,44].

It is now established that PTPN2 is a negative regulator of JAK/STAT signaling, and a negative regulator of the NUP214-ABL1 fusion protein. However, it is hypothesized that PTPN2 can regulate additional signaling pathways in T-ALL. Therefore, to identify additional signaling pathways regulated downstream of PTPN2 we performed phospho-proteomic analysis in the “Mouse Hematopoietic Interleukin-dependent cell line of T-cell Origin” (MOHITO) cell line. The MOHITO cells are a murine CD4+CD8+ double positive T-cell line, extracted from a sublethally irradiated Balb/c mouse, which developed a leukemic disease. The cell line contains both the NOTCH1 and JAK1 mutations, which are typical genetic alterations of the T-ALL disease. The MOHITO cells are dependent on the IL-2 and IL-7 cytokines for cell proliferation by activating the JAK/STAT pathway and as such the MOHITO cell line is considered an ideal cell model to study signaling pathways controlled by PTPN2 in the development of T-ALL[156].

RESULTS

Phospho-proteomics revealed 38 proteins downstream of PTPN2

To identify the signaling pathways governed by PTPN2 we performed phospho-proteomic analysis in MOHITO cells. Therefore, MOHITO cells with knockdown of Ptpn2 and untransduced MOHITO cells were

cultured in growth medium with either heavy or unlabeled arginine and lysine. After cell expansion, cell lysates were made from both cultures and mixed in a 1:1 ratio. By performing immunoprecipitation with two phospho-tyrosine specific antibodies we purified the substrates with tyrosine residue phosphorylation. The purified proteins were separated using gel electrophoresis. After visualization of the proteins with coomassie staining, the gel was cut in small pieces for in-gel digestion with trypsin. Trypsin cleaves peptides at the C-side of arginine and lysine, except when either one is followed by proline, resulting in small peptide fragments. Identification of these fragments occurred in a last step by mass spectrometry. In total, 38 proteins were identified that had 1,5 fold higher phosphorylation in the MOHITO cells with knockdown of Ptpn2 compared to the control cells. We classified these downstream signaling proteins of PTPN2 into five groups according to their known role in T-cell signaling, immunological synapse, proliferation-survival and migration-adhesion as described in publications (Fig. 27A).

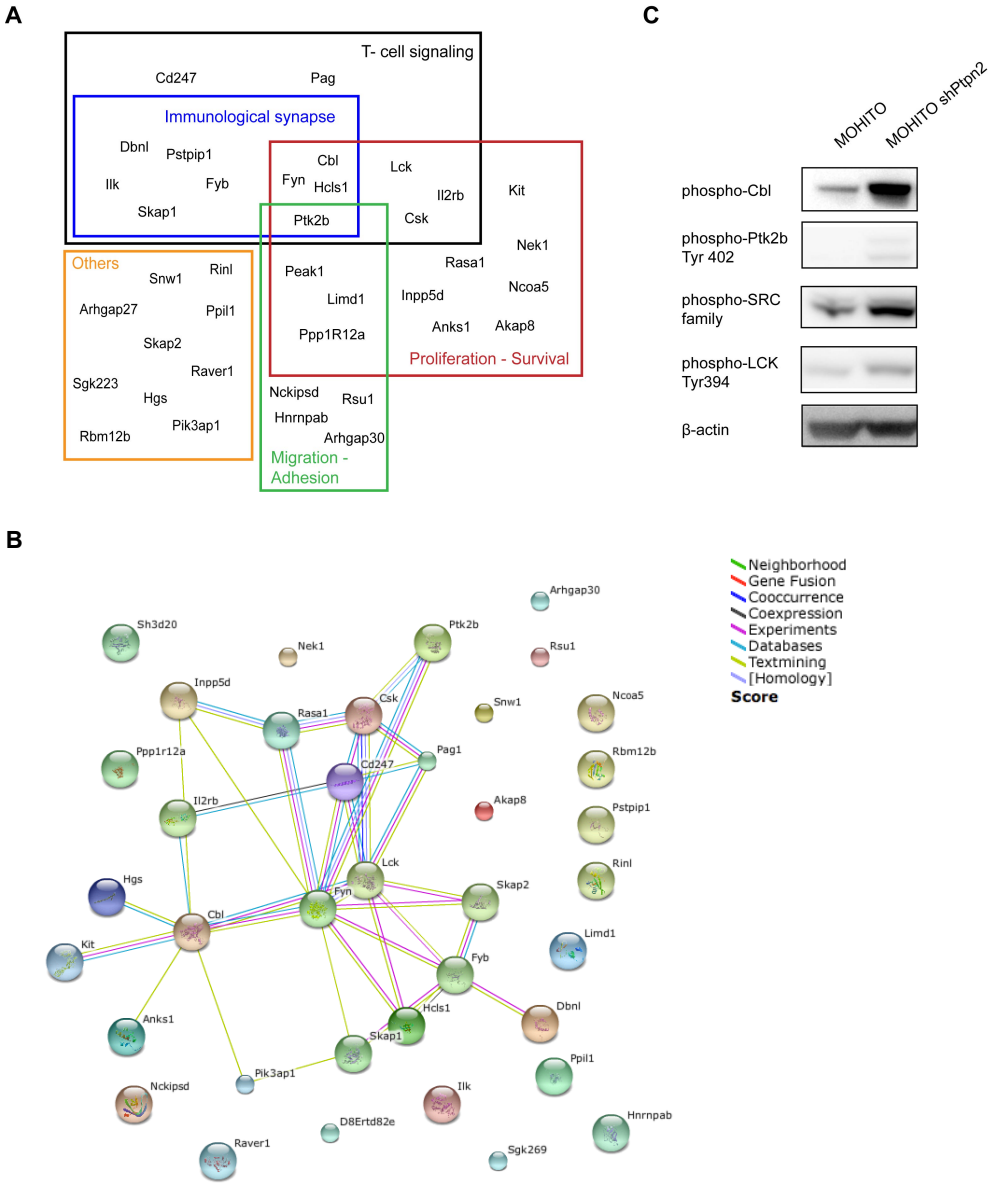


Figure 27. Phospho-proteomics identified 38 proteins downstream of Ptpn2 in MOHITO cells (A) Phospho-proteomics identified 38 proteins downstream of Ptpn2 in MOHITO cells, which are grouped according to their known role in T-cell signaling, formation of immunological synapse, proliferation and survival or migration and adhesion. (B) The STRING analysis visualizes a cluster of predicted protein interactions between proteins involved in T-cell signaling and immunological synapse formation based on information derived from previously reported genomic analysis, high-throughput experiments, co-expressions and publications. (C) Protein-analysis confirmed a higher phosphorylation of Cbl, Ptk2b and the Src family member, Lck in MOHITO cells with shRNA-mediated knockdown of Ptpn2 compared to control cells.

To find out which of the 38 proteins from our phospho-proteomics experiment are already known to interact with each other, we performed a STRING-analysis. STRING stands for Search Tool for the Retrieval of Interacting Genes/proteins and is a database of known and predicted protein interactions. The interactions include direct, physical and indirect, functional associations, which are derived from four sources: genomic context, high-throughput experiments, co-expression and previous knowledge (PubMed). The STRING analysis showed a cluster between the proteins involved in T-cell signaling and immunological synapse formation (Fig. 27B). In that way the STRING-analysis confirmed our phospho-proteomics results by indicating that PTPN2 is involved in several signaling pathways important in T-cell immune response. Defects in these pathways can explain the development of autoimmune diseases in patients with a SNP located upstream of the PTPN2 locus. Since we are interested in the mechanisms underlying the cause of T-ALL, we decided to focus more on signaling proteins involved in cell growth rather than in immune response.

To confirm the phospho-proteomics results, we analyzed the level of tyrosine phosphorylation of proteins from cell lysates of MOHITO cells with Ptpn2 shRNA-mediated knockdown versus control cells. We confirmed a higher tyrosine phosphorylation of Cbl, Ptk2b Tyr402 and Lck Tyr394 in the MOHITO cells with a downregulation of Ptpn2 compared to the control cells (Fig. 27C).

Further in this study we focused on the role of PTK2B, also named PYK2, in the development of T-ALL. The non-receptor tyrosine kinases, the focal adhesion kinase (FAK) and the proline-rich tyrosine kinase 2 (PTK2B) form together the FAK family and link the TCR and growth factor receptor signaling to proliferation, survival, apoptosis and migration[170-173]. After TCR stimulation PTK2B is phosphorylated and activated by FYN, where after PTK2B will modulate the actin cytoskeleton through phosphorylation of paxillin[170,172,174]. Overexpression of PTK2B promoted migration in glioma cells. Beside its role in migration, PTK2B promotes also cell proliferation downstream of the IL-2 and IL-7 receptor[173]. Stimulation of these receptors by their cytokines in T-cells results in a rapid phosphorylation and activation of PTK2B. Downregulation of PTK2B in these cells results in a reduction of cell proliferation[175,176]. The IL7-R/JAK-STAT pathway is an important signaling pathway in T-cell for their proliferation and is often constitutively activated through mutations in the IL7-R, JAK1 or JAK3 proteins in T-ALL[43-45]. RNA-seq analysis by Atak et. al found PTK2B as a new candidate driver gene in T-ALL[177]. Taking these findings together, PTK2B seemed for us an interesting signaling molecule to study its contribution in the development of T-ALL.

Rapid activation of Ptk2b is induced after short-term IL-2 and IL-7 stimulation and loss of PTPN2 extended its return to baseline

It was previously described that IL-2 and IL-7 stimulation of thymocytes results in a rapid phosphorylation of PTK2B[175,176]. After a cytokine starvation period of 3 hours, stimulation of our murine T-cell line MOHITO with IL-2 and IL-7 during several time points resulted as well in rapid activation of Ptk2b. The maximum phosphorylation of Ptk2b was obtained after 10-15 minutes of cytokine stimulation after which the phosphorylation decreased again to baseline, although activation of Jak1 was retained (Fig. 28A and C). We observed also a rapid phosphorylation of downstream signaling proteins of Ptk2b such as Mek1/2 and Erk1/2

(Fig. 28A). This rapid increase in Ptk2b, Mek1/2 and Erk1/2 phosphorylation after cytokine stimulation was also seen in two mouse leukemia cell lines derived from a JAK3 L857Q bone marrow transplant mice (Fig. 28B). Knockdown of Ptpn2 in MOHITO cells resulted in a slower return to baseline of the Ptk2b phosphorylation after IL-7 stimulation compared to the control cells (Fig. 28C). These data indicate that Ptpn2 is a negative regulator of Ptk2b signaling downstream of the IL-7 receptor pathway in T-cells.

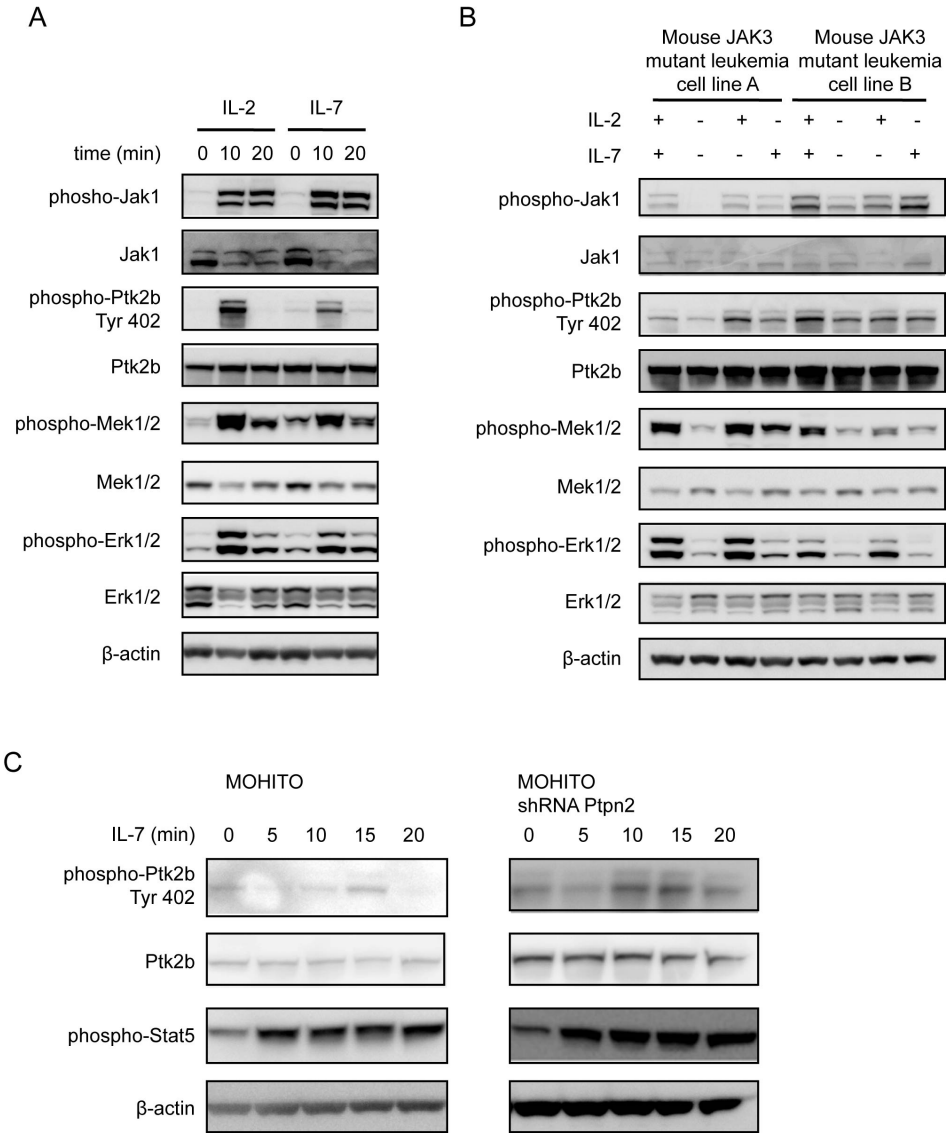


Figure 28. Rapid activation of Ptk2b after IL-2 and IL-7 stimulation in mouse T-cell leukemic cell lines is negatively regulated by PTPN2 (A) Short-term stimulation of MOHITO cells with IL-2 and IL-7 cytokines results in a rapid phosphorylation of Ptk2b and the more downstream signaling proteins, Mek1/2 and Erk1/2, whereas phosphorylation of Jak1 holds on. (B) Also short-term stimulation of two mouse Jak3 leukemia cell lines results in a rapid phosphorylation of Ptk2b and the more downstream signaling proteins, Mek1/2 and Erk1/2. (C) In MOHITO cells, phosphorylation of Ptk2b reaches its maximum by 15 minutes of IL-7 stimulation, where after it returns again to baseline (*left*). A slower return to baseline was observed in MOHITO cells with knockdown of Ptpn2 (*right*).

Src activates Ptk2b downstream of the IL-7 receptor

The PTK2B protein consists of a FERM, kinase and FAT domain. The FERM and FAT domains are involved in the interaction with signaling proteins. The kinase domain is important for the enzymatic activity of the protein after phosphorylation of the tyrosine residue 580 (Fig. 29A). The mechanisms of PTK2B activation differ depending on the activation of the upstream receptors. Activation of cytokine receptors in neuronal,

epithelial and hematopoietic cells results first in Ca^{2+} influx, activation of PI3K or actin remodeling which lead to phosphorylation of the first tyrosine residue of PTK2B, Tyr402. Thereafter, SRC kinases will activate the second tyrosine residue, Tyr580, resulting in enzymatically activation of the PTK2B protein. Instead, activation of the TCR after binding with the major histocompatibility complexes (MHC) of the antigen presenting cell (APC) results first in activation of the SRC kinases LCK or FYN. These SRC kinases will activate the PTK2B protein by phosphorylating the TYR402 and the TYR580 residues (Fig. 29B)[174].

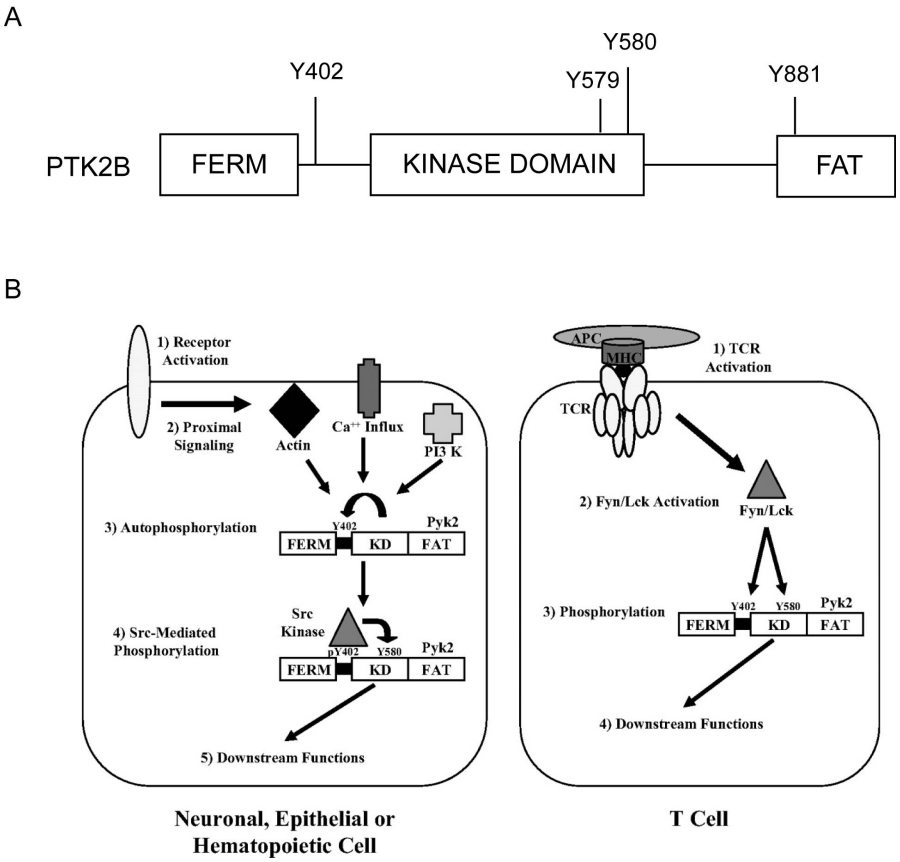


Figure 29. PTK2B structure and regulation (A) Schematic representation of the PTK2B protein, which consist of a FERM, kinase and FAT domain. The four tyrosine residues (Y) are indicated. (B) Regulation of PTK2B, also named PYK2, activation is different downstream of cytokine receptor or TCR receptor. Stimulation of a cytokine receptor causes first a Ca^{2+} influx, activation of PI3K or actin remodeling, which will result in the phosphorylation of Tyr402. Next, the SRC kinase phosphorylates Tyr580 for the enzymatic activation of PTK2B (*left*). Activation of the TCR activates first the SRC kinases, which will simultaneously phosphorylate the Tyr402 and the Tyr580 of the PTK2B protein. (Adapted from reference[174])

We studied how Ptk2b got activated downstream of the IL-7 receptor by performing SRC inhibitor experiments. After treatment with the SRC inhibitors, AZD0530 and dasatinib, Ptk2b phosphorylation was completely blocked even after stimulation with IL-7 in MOHITO cells and in two mouse Jak3 mutant leukemia cell line (Fig. 30A and B). This result showed that Ptk2b phosphorylation downstream of the IL-7 receptor is completely dependent on the Src kinases.

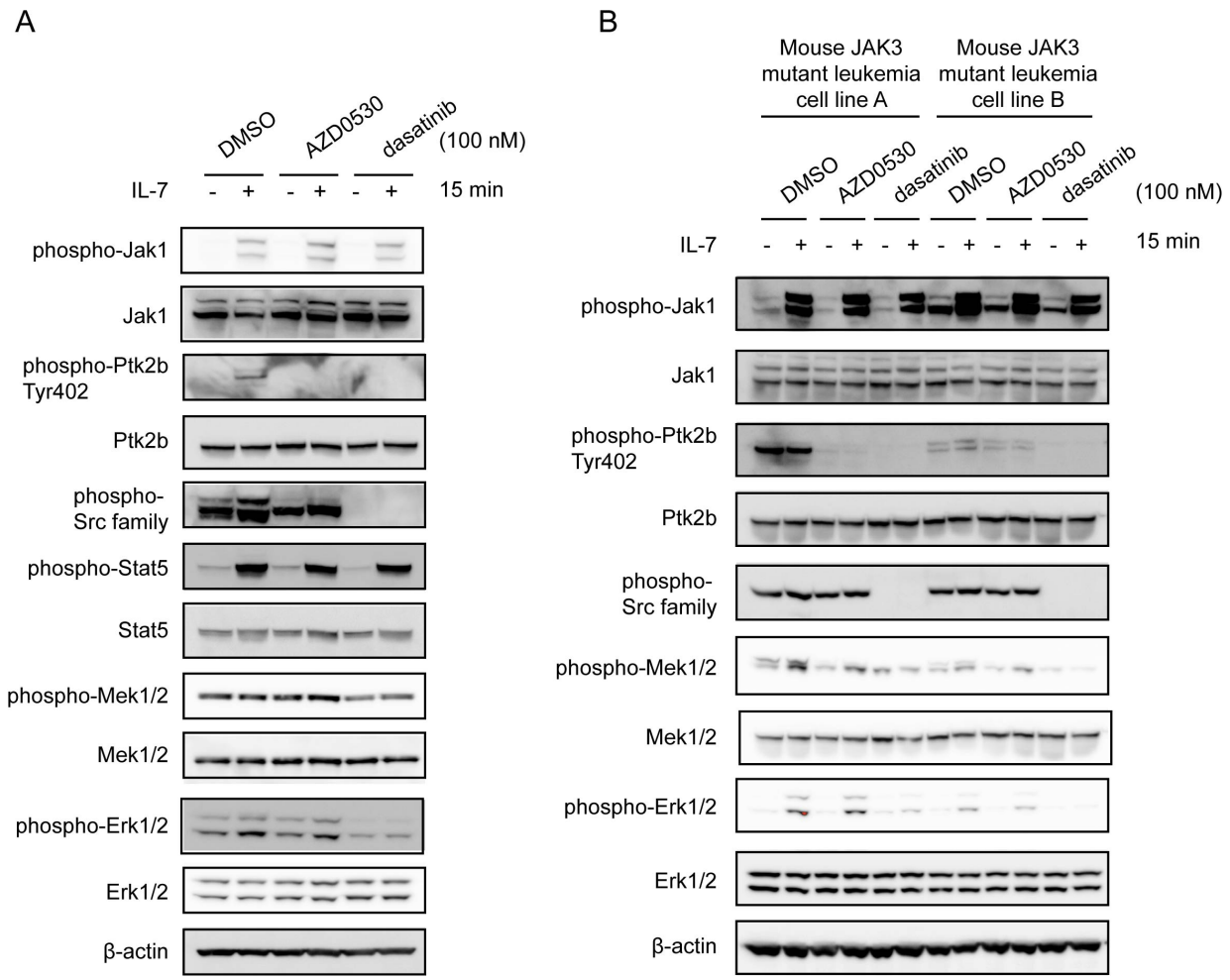


Figure 30. Inhibition of Src kinases blocked activation of Ptk2b (A) Treatment of MOHITO cells with the SRC inhibitors AZD0530 and dasatinib, inhibited Ptk2b phosphorylation after IL-7 stimulation. (B) Also treatment of two leukemic T cells, derived from the Jak3 L857Q bone marrow transplant mice, with the AZD0530 and dasatinib SRC inhibitors blocked phosphorylation of Ptk2b after stimulation with IL-7.

PTK2B is not involved in T-cell proliferation or migration

Since PTK2B is involved in the IL-7R JAK/STAT pathway we assumed that PTK2B would also play a role in T-cell proliferation. However, siRNA-mediated Ptk2b knockdown in MOHITO cells had no effect on their cell growth. A siRNA targeting *Jak1* was used as a positive control, since knockdown of Jak1 results in a reduction of cell proliferation in MOHITO cells (Fig. 31A). Since the IL-7R LSRC, the JAK1 A634D, the JAK3 M511I and JAK3 L857Q mutants transform the MOHITO cells, we wondered whether Ptk2b contributes in this proliferative advantage. However, siRNA-mediated knockdown of Ptk2b did not affect the cell growth of the transformed MOHITO cells. Only knockdown of Jak1 in the transduced MOHITO cells with the IL-7R LSRC and JAK3 M511I mutants caused a decrease in cell proliferation because they are still dependent on Jak1 for their growth (Fig. 31A). Neither inactivation of Ptk2b by treating the MOHITO cells with SRC inhibitors, AZD0530 and dasatinib, had an effect on their growth (Fig. 31B). We supposed that combination treatment of MOHITO cells with SRC and JAK inhibitors would have an effect on cell proliferation, since Ptk2b is downstream of the IL-7R pathways and gets activated by Src. Instead, dose-response curves of the JAK inhibitors ruxolitinib and tofacitinib showed no differences in combination with AZD0530 treatment compared to the DMSO control (Fig 31C).

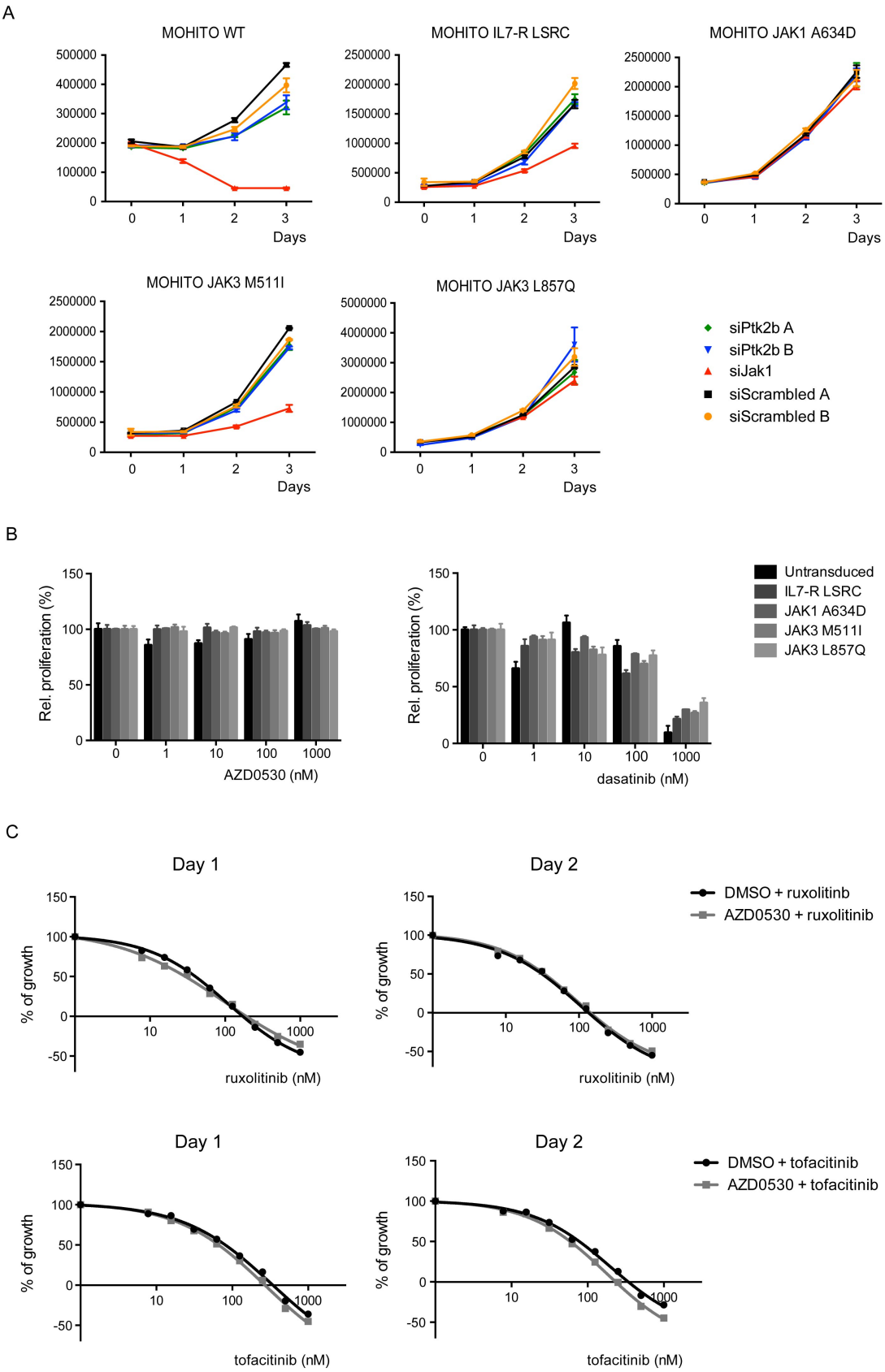


Figure 31. Inactivation of Ptk2b does not affect the proliferation of the T-cell leukemic cell line (A) SiRNA-mediated knockdown of Ptk2b had no effect on the cell proliferation of the untransduced MOHITO cells or the transformed MOHITO cells by the IL-7R LSRC, JAK1 A634D, JAK3 M511I and JAK3 L857Q mutants compared to the scrambled controls. The siRNA targeting *Jak1* was used as a positive control, which reduced the proliferation of the untransduced MOHITO cells and IL-7R LSRC and JAK3 M511I transduced MOHITO cells. Mean growth ± SEM was recorded in triplicate for 3 days. (B) Inactivation of Ptk2b by the SRC inhibitors AZD0530 (*left*) and dasatinib (*right*) had no effect on the cell proliferation of the untransduced and transformed MOHITO cells. Mean growth ± SEM was recorded in fivefold on day 0 and 1. Relative proliferation percentage was calculated by setting cell growth of untreated cells to 100%. (C) Dose-response curves showed no difference in growth percentage of MOHITO cells when treated with AZD0530 in combination with the JAK inhibitors ruxolitinib or tofacitinib compared to the DMSO and AZD0530 as a control. The error bars indicate SEM.

Besides its role in proliferation, PTK2B is known to be involved in cell migration by regulating the actin cytoskeleton. Since we showed that inactivation of Ptk2b had no effect on cell proliferation in MOHITO cells, we investigated its role in cell migration by performing a migration assay. Therefore, we used ThinCerts with a pore size of 8 µm, which were placed in a 24-well plate filled with 20% growth medium. The MOHITO cells, resuspended in 5% growth medium, were added to the ThinCerts. A final concentration of 100 nM AZD0530 and dasatinib were added to the medium of the insert and the well to inhibit Ptk2b activation. After an incubation time of 6 hours the amount of viable cells in the well and the ThinCert was measured to calculate the percentage cell invasion. Their was no difference in the percentage cell invasion between cell treated with the SRC inhibitors compared to the control cells treated with DMSO (Fig. 32). These data indicate that Ptk2b is not involved in the migration of the MOHITO cells.

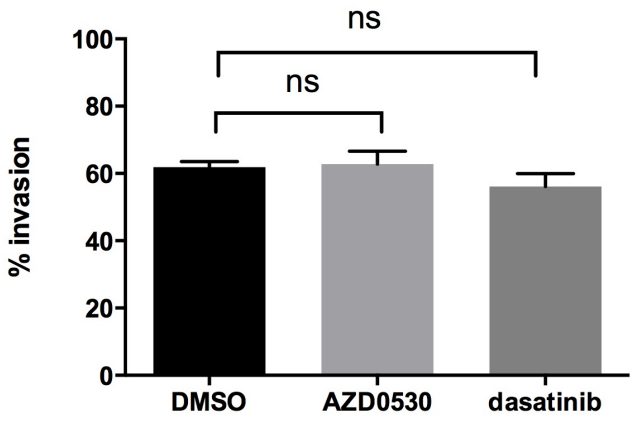


Figure 32. Inactivation of Ptk2b had no effect on the cell migration of the T-cell leukemic cell line The migration assay showed no significant difference (p>0,05) in percentage cell invasion between MOHITO cells treated with the SRC inhibitors AZD0530 and dasatinib compared to the DMSO control. The migration assay was performed in triplicate. The significance was determined by the Mann-Whitney test.

DISCUSSION AND CONCLUSION

Previous studies described that the non-receptor protein tyrosine phosphatase, PTPN2, is a negative regulator of the JAK/STAT pathway. Loss of *PTPN2* by deletions occurred in 6% of the T-ALL cases and was associated with the presence of *JAK1* mutations and *NUP214-ABL1* fusion genes. Loss of the negative regulator PTPN2 increases the oncogenic activity of the JAK1 mutants and the NUP214-ABL1 fusion protein, which enhances the transforming capacity of the leukemic cells. Since the JAK1 mutants and NUP214-ABL1 fusion proteins were present in only a small group of the PTPN2-negative T-ALL cases, other oncogenic kinases are assumed to be involved in the pathogenesis of T-ALL downstream of the PTPN2 phosphatase. In this part of the project we aimed to identify the other signaling pathways controlled by PTPN2 by performing phospho-proteomics in the IL-7 dependent murine leukemic T-cell line, MOHITO, with and without knockdown of PTPN2. Analysis of the phospho-proteomics data identified 38 proteins with a 1,5 fold increase in their phosphorylation when PTPN2 was downregulated in the MOHITO cells. According to their biological function described in research publication we grouped the identified proteins into five classes: T-cell signaling, immunological synapse, proliferation-survival, migration-adhesion and others.

Since PTPN2 plays an important role in immune response it is not so remarkable that a large group of the identified phospho-proteomics proteins are involved in **T-cell signaling** and immunological synapse formation. A first step in the T-cell immune response is the binding of the TCR of the T-cell to the

peptide-major histocompatibility complex (MHC) of the antigen-presenting cell (APC). The TCR consists of a heterodimer with TCRα and TCRβ chains or TCRγ and TCRδ chains, which can bind the peptide-MHC complex, but lacks intracellular signaling transduction motifs. Therefore, the CD3 γ, δ, ε and ζ chains, containing the immunoreceptor tyrosine-based activation motifs (ITAMs), are bound in three dimers (γε, δε and ζζ) to the TCR[178]. The CD3ζ, also called CD247, was one of the phospho-proteomic outcomes. The TCR/peptide-MHC complex is stabilized after binding of the CD4 or CD8 co-receptor with the MHC molecule. The SRC kinases, LCK and FYN, are thereafter recruited which phosphorylate the tyrosine residues in the ITAMs. Subsequent the ZAP-70 kinase is recruited to the TCR complex, where it is phosphorylated and activated by LCK. The ZAP-70 kinase per se will phosphorylate the LAT and SLP-76 adapter proteins, which results on the one hand in cytoskeletal rearrangement and on the other hand in cytokine production, proliferation and survival by regulating gene transcription through activation of the RAS pathway and mobilization of Ca^{2+}. In this way TCR signaling enhances and maintains the T-cell immune response[179-182].

Not so surprising is the presence of the SRC family kinases, FYN and LCK, in our phospho-proteomics data, since PTPN2 is a known negative regulator of the TCR signaling by dephosphorylating LCK. The increased LCK activation, due to the Ptpn2 deficiency in the naïve T cells from the *Lck*-Cre;*Ptpn2*$^{fl/fl}$ mice, enhanced the TCR activation and proliferation and lowered the TCR threshold response to low-affinity antigens. These increased T-cell responses to low-affinity ligands may explain the inflammatory and autoimmune phenotype in the *Lck*-Cre;*Ptpn2*$^{fl/fl}$ mice[122]. The SRC family kinase are not only important during TCR signaling, but also at several stages of T-cell development, such as pre-TCR signaling, positive selection and homeostasis of naïve T-cells[180].

The IL-2 cytokine production after T-cell activation is an important T-cell immune reaction. IL-2 is secreted by the T-cell and binds to the IL-2 receptors present in the membrane of the same T-cell to promote clonal expansion and acquisition of effector or memory function. The IL-2 receptor consists of the α, β and γ chain. The JAK1 kinase is bound to the IL-2 receptor β chain and the JAK3 kinase to the IL-2 receptor γ chain. The IL-2 receptor β chain (or IL2Rb) appeared in our phospho-proteomics results[182].

A negative regulator of the T-cell receptor signaling is the C-terminal SRC kinase (CSK). CSK phosphorylates the C-terminal tyrosine residue of LCK and FYN, which results in an inactive confirmation of the enzyme. CSK is located in the proximity of the SRC kinases through its association with the lipid raft targeted transmembrane adapter protein, PAG. The CSK-PAG binding is possible because of the phosphorylation of PAG by FYN, showing that the SRC family kinases regulate their own activity. After TCR stimulation PAG is dephosphorylated, resulting in the release of CSK and which favor LCK and FYN activation. CSK and PAG were present in our phospho-proteomics data[179-181].

Binding of the TCR with the peptide-MHC complex results in the formation of an **immunological synapse** between the T-cell and the APC. After stimulation of the TCR, the TCR signaling cascade induces remodeling of the cytoskeleton to cluster integrins around the TCR complex. In that way the immunological synapse is stabilized to sustain T-cell activation and proliferation. The process of remodeling the cytoskeleton involves a cluster of signaling pathways of which some signaling molecules appeared in our phospho-proteomics dataset, such as FYN, FYB, PTK2B, PSTPIP1, HCLS1, SKAP1 and ILK[170,179,183-187].

Two negative regulators, DBNL and CBL, of the immunological synapse and the TCR signaling cascade were also present in our dataset. DBNL is part of the immunological synapse and reduces TCR expression by gene regulation via the NFAT transcription factor[188]. CBL inhibits the formation of the immunological synapse by repressing TCR activation in the absence of the CD28 co-stimulator[189].

The third group of our phospho-proteomics data contains the proteins involved in **proliferation and survival** either in positive or negative way. The receptor tyrosine kinase, KIT, has an important role in the maintenance and survival of hematopoietic stem cells and is negatively regulated by CBL[190]. It is a known oncogene in cancer, such as acute myeloid leukemia. The serine/threonine kinase, NEK1, is one of the eleven members of the NIMA-related kinases or NEK-family, which are important during mitosis. NEK1 is involved in sensing and repairing DNA strand breaks at the G1-S1 and G2-M transition[191]. RASA1 is a repressor of the RAS pathway and controls in that way cell proliferation. NCOA5 is a nuclear receptor co-regulator with activating and repressive function. The protein is described as a tumor suppressor in hepatocellular carcinoma in type II diabetes[192]. The anchoring protein, AKAP8, is responsible for the PKA compartmentalization and is in that way important during cell cycle[193]. INPP5D is a member of the inositol polyphosphate-5-phosphatase (INPP5) family. After phosphorylation it translocates from the cytosol to the membrane where it negatively regulates the PI3K pathway, and so proliferation, through hydrolysis of phosphatidylinositol-3,4,5-trisphosphate to phosphatidylinositol-1,3,4,5-tetrakisphosphate[194]. ANKS1 is a negative regulator of growth factor signaling by degradation of growth receptors through endocytosis[195].

In a fourth group we divided proteins known to be involved in **cell adhesion and migration**. The adaptor protein NCKIPSD stimulates WASP induced ARP2/3 complex formation and influences in that way the actin polymerization and so cell adhesion[196]. ARHGAP30 is a RhoGTPase activating protein involved in cell adhesion and migration[197]. Overexpression of the transcriptional regulator HNRNPAB results in an increased expression of SNAI1 and so in an increase in cell invasion[198]. The RAS repressor, RSU1, inhibits the PINCH1-ILK complex, which is involved in cell migration and upregulated in some tumors[199]. The tumorsuppressor LIMD1 is a transcriptional regulator that inhibits E2F transcription through interaction with retinoblastoma. Decrease of the expression of E2F target genes results in a reduced cell migration and proliferation[200]. PPP1R12A, is one of the subunits of the myosin phosphatases and is involved in cell division and migration through its regulation of the myosine phosphatase activity[201]. The atypical kinase PEAK1 (also named SGK269) is described to play a role in cell invasion and migration in breast cancer through changing phosphorylation of cytoskeleton-associated proteins MAPK1/ERK and paxillin[202].

The fifth group “Others” contains the phospho-proteomics results that could not be classified directly in the four other groups. This group contains the SNW1, RAVER1 and PPIL1 proteins involved in mRNA splicing and the RBM12B protein with a RNA binding motif of which the function is not yet clear[203,204]. RINL is involved in intracellular membrane trafficking by regulating endocytosis[205]. The RhoGTPase activating protein, ARHGAP27 (also named SH3D20), plays a role in clathrin-mediated endocytosis. SGK223 (also named D8ERTD82E) is involved in the regulation of neurite outgrowth[206]. HGS recycles and degrades receptors by lysosome-dependent degradation[207]. The PIK3AP1 and SKAP2 proteins are part of the B-cell receptor signaling in B-cells[185,208].

Since we aimed to identify the signaling pathways contributing to the pathogenesis of T-ALL, we are interested in the group of proteins involved in proliferation, survival and migration. In particular, we focused on the tyrosine kinase PTK2B, because it is a known downstream signaling protein of the IL-2 and IL-7 receptors and which is involved in cell proliferation and migration dependent on the cellular context. Atak et.al identified PTK2B as one of the new candidate driver genes in T-ALL after analysis of RNA-seq data in T-ALL samples[177]. Our goal was to identify the biological role of PTK2B in the development of T-ALL. We used the MOHITO cell line as model for our study because of its T-ALL characteristics, such as having Notch1 and Jak1 mutations, their dependency on IL-2 and IL-7 and the Jak/Stat pathway as the driving force for their proliferation. We confirmed the induction of rapid phosphorylation of Ptk2b after stimulation of MOHITO cells with IL-2 and IL-7. The activation of Ptk2b was completely dependent on the Src kinases and was negatively regulated by Ptpn2. Although Benbernou et al. described a block of proliferation in the IL-7 dependent murine thymocyte cell line, D1, after targeting Ptk2b with an antisense oligonucleotides, we could not confirm that Ptk2b was involved in the cell proliferation of our T-cell leukemic cell line[175]. Neither siRNA-mediated downregulation of Ptk2b nor inactivation of Ptk2b using SRC inhibitors had an effect on the cell proliferation of the MOHITO cells. Since Ptk2b had no influence on the cell proliferation of the MOHITO cells, we investigated its role in the cell migration of these cells[209]. However, inactivation of Ptk2b by SRC inhibitors showed no difference in migration compared to control. Probably PTK2B is more involved in migration downstream of the TCR as described before[170,172]. Our functional data indicates that PTK2B is not an important driving force in the development of T-ALL.

CHAPTER V – GENERAL DISCUSSION

Eukaryotic cells use protein tyrosine phosphorylation as a mechanism (i) to regulate signal transduction pathways, (ii) to communicate with neighboring cells and (iii) to transfer intracellular signals to induce a cellular response after an initial stimulus. The regulation of protein tyrosine phosphorylation is under tight control of PTKs and PTPs whereby PTKs phosphorylate proteins on tyrosine residues and PTPs counteract this action by removing the phosphate group. In general PTKs activate signaling pathways whilst PTPs are responsible for their inactivation, although exceptions to this rule are known to exist. This makes the PTKs and PTPs important regulators of cellular responses such as proliferation, survival, differentiation, metabolic regulation, motility, adhesion and migration. Not surprisingly, interruption of this tightly regulated balance between PTKs and PTPs contributes to the development of human diseases, including cancer. Genetic and epigenetic alterations affecting the PTK and PTP genes often account for the underlying cause of this protein tyrosine phosphorylation imbalance. These alterations can alter the enzyme activity, the subcellular localization or the expression levels of the PTKs and PTPs with severe consequences for the cell.

Over the past few decades the role of PTKs in the development of cancer has been studied intensively. Although the PTKs form a small subgroup with 90 members out of the 518 proteins belonging to the protein kinase superfamily, this subgroup is more frequently mutated in cancer. This underlines again the importance of PTKs in the regulation of cellular functions. Numerous research studies investigating the mechanisms of oncogenic PTKs in cancer has also revealed insights into how PTKs are regulated and in which signaling pathways they are involved. This knowledge led to the development of promising PTK inhibitors with a higher specificity and less toxicity compared with many current chemotherapeutic agents.

In the cancer research the use of cultured cancer cell lines have become useful models to perform experimental studies when there is restricted availability and limited supply of primary patient samples. These cancer cell lines are used to characterize the genetic background of the cancer from which they originate, to study signaling pathways involved in the tumor development, to perform RNAi and drug screens and to set up drug-resistant profiles. Although these cell culture lines are useful model systems, it is important to bear in mind they have some shortcomings. Cell culture lines are having genome instability and their long-term culture results in newly acquired genetic alterations to increase the genomic complexity as well as selection of a clonal tumor subpopulation. Cultured cancer cell lines also lack their natural microenvironment, which can have an influence on their behavior. Therefore, it is important that any findings found in cell culture lines are also confirmed with relevant primary cancer samples.

Our laboratory has experience in using cytokine-dependent leukemic mouse cell lines for transformation studies and drug screens. In the lab, our cell line models of choice are the murine IL-3 dependent pro-B-cell line Ba/F3 and the IL-2 and IL-7 dependent T-cell line MOHITO. Overexpression of a mutant PTK with constitutive enzymatic activity will drive the proliferation of the cells independently of the cytokine signaling pathway. This makes the cytokine-dependent leukemic cell lines an interesting model to study the transforming capacity of newly identified PTK mutants, but also to test the sensitivity and specificity of newly developed compounds directed against the oncogenic PTKs. Treating the cells with drug compounds targeting the oncogenic PTKs will result in a reduction of the cell growth in the absence of the cytokine.

We used the transforming capacity of oncogenic tyrosine kinases in the IL-3 dependent cell line Ba/F3 to develop an RNAi screening system in selecting effective shRNA and siRNA molecules. RNAi is a process of gene silencing, whereby short RNA molecules target the mRNA in a sequence-specific manner for degradation. The synthesized RNAi molecules, shRNA and siRNAs, are used in the research to experimentally knockdown the expression of genes in cell lines or in cells *in vivo* and became interesting tools in therapeutic approaches. Besides difficulties with delivery and specificity, another major challenge of using RNAi molecules is selecting effective shRNAs or siRNAs that sufficiently downregulate the expression of the target gene. Although algorithms to design RNAi molecules have been improved lately, validation is still required before using them in experimental and therapeutic approaches. To this end, we developed a simple cell-based system in which the effect on proliferation of the Ba/F3 cells is used as read-out to select effective RNAi molecules. Specifically, our model Ba/F3 cell system overexpress a truncated form of PDGFRα, which drives their proliferation in the absence of IL-3. The transcript of the truncated PDGFRα is linked to a gene of interest. Introduction of shRNAs and siRNAs that effectively target the gene of interest will also lead to the degradation of the mutant PDGFRα transcript, which is detectable because of the block in cell growth. The cell proliferation as a primary end point read-out has an important advantage, since it is hard to predict in advance which reduction in protein level is required to have a cellular response. Furthermore, the Ba/F3 cells also have a rapid doubling time and can be transfected and transduced efficiently. Toxic effects caused by the RNAi molecules can be easily detected by rescuing the proliferation block through the addition of the growth factor IL-3. Another advantage, compared to other RNAi screens, is the independency in obtaining high transduction efficiencies. The shRNA constructs contain a GFP marker gene, which makes it possible to follow the cell proliferation according to the percentage of GFP positive cells.

It was initially believed that PTPs were just housekeeping genes, which simply balance the effects of the PTKs. Recent functional studies and genetic screens indicate that PTPs are active regulators, which contribute in addition to PTKs, to the development of several cancer types. However, compared to the PTK research, studies of PTPs are lagging behind and many questions remain concerning their regulation, biological function and mechanism. The aim of the second part of my thesis was to study the role of PTPRD as a candidate tumor suppressor in the development of cancer, because gene inactivation through mutations, deletions and promoter methylations was frequently reported in different solid tumors[66,68-70,103]. Also our CNV analysis revealed a high frequency of *PTPRD* deletion in T-ALL and B-ALL samples. The *PTPRD* gene is located on the short arm of chromosome 9 in close proximity to *CDKN2A*, which is a well-documented tumor suppressor frequently lost in tumors. The short arm of chromosome 9 is often subjected to loss-of-heterozygosity in cancer, which could only be partially explained by loss of *CDKN2A* locus. Therefore, researchers postulated the existence of a second tumor suppressor gene on 9p[69,70,210]. Because of the high frequency of PTPRD inactivation in many cancers, PTPRD appeared to be a good candidate to fulfill the role as this second tumor suppressor next to CDKN2A. Veeriah et al. showed that loss of *PTPRD* is not driven by *CDKN2A* loss because different mechanisms, such as methylation and CNV, were responsible for the inactivation of these two loci[70]. Instead, we observed a simultaneous loss of *PTPRD* and *CDKN2A* due to deletions of larger parts of 9p in almost all of the ALL samples. This questions the tumor suppressive role of PTPRD in ALL, especially because we did not observe a correlation between the PTPRD gene expression levels and its CNV in the B-ALL samples.

Another observation is the loss of only one copy of *PTPRD* in all our analyzed ALL samples. Unlike *CDKN2A*, which is most common inactivated by one large deletion and one focal deletion or mutation of the second allele, *PTPRD* seems not to be homozygously inactivated. From our mutation analysis we can conclude that point mutations are not contributing to the inactivation of PTPRD, since the frequency is extremely low. However, it is still possible that PTPRD acts as a haploinsufficient tumor suppressor. The concept of haploinsufficiency is in sharp contrast with the two-hit hypothesis since it premises that even partial loss of a tumor suppressor gene is sufficient to contribute in tumorigenesis. Haploinsufficiency has already been shown previously for the tumor suppressor genes *PTEN* and *TP53*, whereby the function of the gene is coupled to the change in its expression level or protein activity[166]. Recently, Ortiz et al. observed that haploinsufficiency of the *Ptprd* gene promoted tumor progression in their GBM mouse model in cooperation with *Cdkn2a* deletions. Most GBM samples had focal deletions of the *PTPRD* locus in co-occurrence with focal deletions of the *CDKN2A* gene. In 87% of the samples with *PTPRD* deletions only one copy was lost. Heterozygous loss of *Ptprd* together with homozygous deletion of *Cdkn2a* in the GBM mice was more efficient in promoting tumorigenesis compared to wild type *Ptprd* and homozygous loss of *Ptprd*. This heterozygous loss of *Ptprd* concurrent with *Cdkn2a* loss was accompanied with an increase in pStat3. Although Stat3 is an important transcription factor in many proliferation pathways and previous functional data found *Ptprd* to be a growth inhibitor, the tumor progression in these mice was not caused by increased cell proliferation. Instead, $Ptprd^{+/-}p16^{-/-}$ had enrichment in pathways involved in immune response and macrophage behavior[113]. Genetic analysis only in human cancer samples is not enough to define the haploinsufficiency of a tumor suppressor gene. Therefore, more *in vitro* and *in vivo* experiments are required to define the haploinsufficiency of PTPRD in other cancer types. Even though the role of PTPRD as a potential tumor suppressor in ALL is inconclusive, the extramedullary hematopoiesis observed in the *Ptprd* knockout mice and the PTPRD expression in human hematopoietic stem cells and progenitor cells suggest a potential role of PTPRD in hematopoiesis. More research is needed to define whether the extramedullary hematopoiesis is caused by defects in hematopoietic cells or in tissue cells of spleen or bone marrow. Understanding the biological role of PTPRD in hematopoiesis could help to gain more insights in its possible role in hematological malignancies.

To determine whether our observations in ALL also correlate with solid cancers where PTPRD has already been shown to be a tumor suppressor, we extended our studies into melanoma. According to our results, the tumor suppressive role of PTPRD in melanoma is also questionable despite of the high mutation rate. Indeed, we observed a very low expression level of PTPRD in human and murine melanoma samples as well as in their normal cells of origin, the melanocytes. Moreover, the described mutations of the *PTPRD* locus seem to be introduced at a random basis, with mutations scattered the entire length of the gene and with a higher frequency in the introns than in the exons. As such, we hypothesize that the majority, if not all, mutations are passenger mutations, most likely introduced by UV radiation evidenced by the high frequency of C to T transitions. This hypothesis questioning the role of PTPRD as a bona fide tumor suppressor has also been brought into question by Clark et al. in neuroblastoma due to the very low PTPRD expression both in the neuroblastoma cells and the mouse embryo adrenal glands, the cells of origin of neuroblastoma. They could also not confirm a growth inhibition after reconstitution of PTPRD in neuroblastoma cell lines[158]. Although Solomon et al. have shown that PTPRD is involved in growth reduction after reconstitution of PTPRD in melanoma and GBM cells, these results are not indicative for the same biological function of the

endogenous protein in these cancer cells[69]. The same conclusion can be made for the STAT substrates of PTPRD, so far only identified in 293T cells. The 293T cell line is not a representative model for the cancers, and therefore STAT1 and STAT3 still need to be confirmed as substrates of PTPRD in the cancer cells.

Last years with the increasing use of high-throughput screening methods, genetic data are produced in massive amount, which results in the discovery of many novel genetic alterations in different cancer types. In that way genetic alterations in *PTPRD* were observed in many different cancer types. However, the occurrence of mutations in specific genes is not sufficient to establish their role as tumor suppressor genes, and our data strongly suggest that PTPRD is not relevant in melanoma development. Loss of PTPRD may contribute to the development of other cancers, and it will be of interest to study a possible tumor suppressive role of PTPRD in lung, colorectal and head and neck cancer, since PTPRD deletions and promoter methylations were found in these cancer types. In a first step the expression levels of PTPRD in the healthy tissues and the cancer samples should be defined. When the phosphatase is expressed in the healthy tissues and the expression levels in the cancer samples correlates with the genetic data, the biological function of PTPRD should be further investigated in cancer cell lines. Its role in cell proliferation could be studied by performing proliferation and apoptosis assays in cancer cell lines after RNAi-mediated knockdown. It would be interesting to investigate whether STAT1 and STAT3 are downstream signaling proteins in these cancer cell lines. Phospho-proteomics is a useful technique to identify other signaling pathways controlled by PTPRD, which would contribute to the understanding of the biological role of PTPRD in these cancer types. As shown by Ortiz et al. in GBM, a possible cooperation of loss of *CDKN2A* and *PTPRD* should be studies as well in other cancers with high frequencies of deletions of these loci. From this part of the thesis, we can underline the importance of performing gene expression analysis and functional studies in cell lines as well as in mouse model and patient samples, in addition to the genetic analysis of the cancer samples.

In the last part of my thesis, we used phospho-proteomics to identify other signaling pathways controlled by PTPN2 in leukemic cells. Previously, the JAK/STAT and NUP214-ABL1 proliferation pathways are described to be negatively regulated by this non-receptor protein tyrosine phosphatase in T-ALL. However, other signaling pathways involved in T-ALL and under control of PTPN2 remained unknown. Phospho-proteomics is an interesting high-throughput screening system to study the difference in protein tyrosine phosphorylation between cell lines with and without a PTP. Analysis of our phospho-proteomics data identified 38 proteins with a higher phosphorylation level on their Tyr residue in the leukemic T-cell line, MOHITO, with knockdown of PTPN2 compared to control. The identification of signaling proteins involved in T-cell signaling and immunologic synapses confirmed the role of PTPN2 in immune response and auto-immune diseases. Also many signaling proteins involved in cell proliferation and migration are under control of PTPN2, including PTK2B, which we studied in more detail. Although Ptk2b functions downstream of IL-7R pathway, which drives the proliferation in MOHITO cells, Ptk2b was not involved in the cell proliferation or migration of these cells. Interestingly, PTK2B also functions downstream of the TCR, where it modulates the actin cytoskeleton. In T-cells, the IL-7R pathway maintains their homeostasis, whereas the TCR signaling pathway induces an immune response after antigen-induced stimulation. During this TCR signaling, the IL-7R pathway is inhibited by a change in the balance of pro- and anti-apoptotic proteins. However, the exact mechanism

behind this switch in signaling proteins is unknown. PTK2B might be involved in this crosstalk between these pathways[211,212].

Although we could not provide evidence that PTK2B is contributing to the pathogenesis of T-ALL, the phospho-proteomics identified several other proteins downstream of PTPN2 that might be involved in the development of T-ALL. The KIT tyrosine kinase, for example, appeared also in the phospho-proteomics data and has a known role in cell proliferation. The KIT protein is a proto-oncogene in acute myeloid leukemia, but is also expressed in some T-ALL patient samples. Therefore, researchers have described KIT as a candidate oncogene in T-ALL, although its transforming capacity in this malignancy has not been investigated in great detail[213-215]. For the other identified proteins involved in cell proliferation (CBL, NEK1, RASA1, NCOA5, AKAP8, INPP5D and ANKS1) there were no genetic analyses or functional data published that indicate their potential role in the pathogenesis of T-ALL. It would be of interest to perform genetic analyses of T-ALL datasets to define the presence of genetic alterations in these genes. The phospho-proteomics revealed also many proteins involved in T-cell signaling and formation of immunological synapses. Investigating the role of PTPN2 in these signaling pathways could contribute to our understanding of the role of PTPN2 in the pathogenesis of autoimmune diseases.

The question remains why PTP research is still lagging behind that of PTKs. One reason may be that the active role of PTPs in regulating the tyrosine phosphorylation was long underestimated. PTPs were long seen as housekeeping enzymes maintaining the basal level of tyrosine phosphorylation. Recently, genetic analyses revealed more and more gene alterations of PTPs in cancer samples, indicating their active contribution in the carcinogenesis. Functional studies of the oncogenic capacity of PTKs are mostly more straightforward since activating mutations or PTK overexpression results in an obvious difference in, for example, cell proliferation. Loss of tumor suppressive PTPs require in most cases at least two hits for complete inactivation, which complicates and extends the genetic analyses. In case of haploinsuffiency, it is even hard to define which level of protein reduction is causing the phenotype. Moreover, very often loss of a PTP is compensated by other PTPs. For example the PTPRD family members LAR and PTPRS can compensate for its loss in neuronal biology[87,98]. Furthermore a PTP can have more than one function depending on the cellular context. In T-cells, PTPN2 is involved in controlling the immune response as well as their cell proliferation by regulating different signaling pathways. More striking, the same PTP can act either as an oncogene or a tumor suppressor depending on the malignancy. Again the example of PTPN2, which loss through deletions contributes to the development of T-ALL due to increased activation of the JAK/STAT pathway[43,44]. In contrast, elevated levels of PTPN2 were observed in activated B-cell-like diffuse large B-cell lymphoma. The elevated expression of PTPN2 was associated with a tumor-promoting effect through indirect inhibition of IL-4 mediated cell cycle arrest caused by dephosphorylation of STAT6[216]. The fact that not a lot of PTP ligands and substrates are identified is also impeding this research in understanding the biological role of PTP and their contribution to the development of human diseases, such as cancer.

CHAPTER VI – REFERENCES

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Education

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Extra courses

2011	Statistical thinking and reasoning in experimental research - VIB, Gent
2011	Certificate Laboratory Animal Science Module II, KULeuven
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Publication

Pieraets, S., Cox, L., Gielen, O. & Cools, J. Development of a siRNA and shRNA screening system based on a kinase fusion protein. *RNA* **18**, 1296–1306 (2012).

Posters and presentations

Poster 2014	Oncoforum, Leuven Title: PTPN2 regulates PYK2 phosphorylation downstream of IL-7 and IL-2 receptor
Presentation 2013	VIB-NERF Symposium Title: Characterizing the tyrosine phosphatase PTPRD and its role in the development of cancer
Poster 2013	VIB seminar, Blankenberge Title: Development of a siRNA and shRNA screening system based on a kinase fusion protein

Conferences

2013, 2014	VIB-NERF Symposium
2011, 2012, 2013, 2014	Oncoforum
2011	Congress of EHA
2011	VIBes in Biosciences

IT experience

General software	Windows XP and Vista Microsoft Office Mac OS X Adobe Illustrator Adobe Photoshop
Specialized software	GraphPad Prism 6 Flow Jo CLC Workbench Agilent Genomic Workbench Biogazelle qBase+ Papers2

Extra curricular activities

2013	Organisation CME departmental staff party – KULeuven
2011, 2012, 2013	Scientist at work
2012	Guide at Biotech Day Flanders

Hobbies

1999 – now	Volleyball

LIST OF PUBLICATIONS

Pieraets S., Cox L., Gielen O., Cools J. Development of a siRNA and shRNA screening system based on a kinase fusion protein. *RNA* 18, 1296-1306 (2012).